ACTIVITY AND REGULATION OF AGC KINASES FROM *Physcomitrella patens* AND TOMATO

A Dissertation

by

ANNA C. NELSON DITTRICH

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2012

Major Subject: Biochemistry

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Approved by:

Chair of Committee,	Timothy Devarenne
Committee Members,	Mary Bryk
	Hisashi Koiwa
	Pingwei Li
Head of Department,	Gregory Reinhart

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ABSTRACT

Activity and Regulation of AGC Kinases from *Physcomitrella patens* and Tomato.

(August 2012)

Anna C. Nelson Dittrich, B.S., Southwestern Oklahoma State University

Chair of Advisory Committee: Dr. Timothy Devarenne

The AGC group of protein kinases (named for protein kinases A, G, and C) is found in all eukaryotes studied so far, and its members coordinate essential cellular processes including translation, metabolism, hormone response, growth, and survival. AGC kinases are intensively studied in mammals because of their connection with human diseases like cancer, diabetes, and neurological disorders. Some aspects of AGC kinase function are organism-specific, but others are conserved in highly divergent species. Several AGC kinases are regulated by the conserved 3-phosphoinositide dependent protein kinase-1 (PDK1), which is itself an AGC kinase. PDK1 regulates its substrates through phosphorylation at a conserved site in their activation loop.

Here, I identify and characterize a PDK1 homologue from the moss *Physcomitrella patens* (*Pp*PDK1). I show *Pp*PDK1 phosphorylates plant AGC kinases in the activation loop, but unexpectedly lacks a lipid-binding domain, suggesting that its regulation differs from other species. In contrast to mammalian cells, *Pp*PDK1 is not an essential gene, suggesting that AGC kinase pathways in *P. patens* are sufficient for survival even in the absence of activation by *Pp*PDK1. I analyze putative PDK1

sequences from 100 different eukaryotic species, finding that many PDK1s differ from the “conventional” PDK1 found in humans. Phylogenetic analysis of these sequences suggests a complicated evolutionary history for PDK1, with the potential for unexpected functional and regulatory features. I also investigate the regulation of Adi3, an AGC kinase from tomato, through phosphorylation by PDK1. I identify a novel putative PDK1 phosphorylation site outside the kinase domain, which appears to increase Adi3 activity on a substrate. Finally, I produce a mutant version of Adi3 that can selectively utilize bulky ATP analogues. This analogue-sensitive protein may be used in a future search for direct Adi3 substrates.

Together, my experiments provide insight into two members of the AGC group of protein kinases, one (PDK1) that is conserved in all eukaryotes and one (Adi3) that appears to be present only in plants. These experiments give a new perspective in our view of plant AGC kinase function and regulation.

DEDICATION

To my husband Andrew, who has suffered through graduate school with me, been patient with me, kept me somewhat sane, convinced me not to quit numerous times, and most importantly, cared for me outside of work. I never could have done this without your love and help. You are the best!

To my family: my mom Diane, dad Ovie, stepmom Barbara, and brother Josiah. You have always loved me unfailingly and been proud of me regardless of what I do. Thank you so much for your support. I love you all!

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The Biochemistry Graduate Association is a superb organization and resource for all graduate students in the department. The BGA provided me with a mentor to help me adjust to graduate life during my first year, allowed me the opportunity to give a formal presentation at the student research competition during my second year, and helped fund my trip to a scientific conference during my fifth year. My sincere thanks are due to all the people who make the BGA possible.

I had the opportunity to work as a graduate TA for Dr. Bryant Miles, Dr. Leisha Mullins, Dr. William Park, and Dr. Donald Pettigrew. I thank all of them for being great teachers and mentors, and for increasing my knowledge of biochemistry. When I teach in the future, I will surely do a better job because of my experiences working for them.

I am indescribably grateful to Dr. Noah Whiteman and Dr. Mark Beilstein at the University of Arizona, who have given me a fantastic reason to finish graduate school. I look forward to a future of interesting and enjoyable research with them.

Finally, my years as a graduate student would have been unbearable in the absence of friends with whom I could celebrate good times and commiserate during bad times. Becki Murphy and Andrew Nelson were indispensable in this respect. I feel very lucky to be part of their lives, and their support, encouragement, and friendship deserve my endless gratitude.

NOMENCLATURE

AGC	Group of protein kinases named for protein kinases, A, G, and C
PDK1	3-phosphoinositide-dependent protein kinase-1
Adi3	AvrPto-dependent Pto-interacting protein 3
PtdIns(3,4,5)P_3	phosphatidylinositol-3,4,5-trisphosphate
PtdIns(3,4)P_2	phosphatidylinositol-3,4-bisphosphate
PtdIns(4,5)P_2	phosphatidylinositol-4,5-bisphosphate
PA	phosphatidic acid
PH	pleckstrin homology

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW*

1.1 Highlights in the history of protein phosphorylation

1.1a Initial reports of protein phosphorylation: 1950s

The collective efforts of numerous scientists from the 1950s to the 1980s established that protein phosphorylation cascades are reversible, can affect different protein function in different ways, and are sometimes complex. The enzyme-mediated addition of phosphate to a protein was first described almost 60 years ago, with the finding that rat liver mitochondrial extracts are capable of phosphorylating casein at serine residues (Burnett and Kennedy, 1954). This early report established several key features of a protein kinase that are now known to be generalizable to many protein kinases: 1) that a divalent cation (in this case Mg^{2+}) is required for kinase activity, 2) that ATP is the phosphate donor, 3) that an intact protein, rather than a free amino acid, is the substrate of protein kinases, and 4) that protein kinases often display promiscuous activity and can phosphorylate non-physiological substrates. Despite these profound insights into the nature of protein kinases, the authors did not fully understand the

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This dissertation follows the style of Plant Physiology.

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*Portions of the following articles have been reprinted with permission: (1) **Dittrich ACN, Devarenne TP** (2012) Characterization of a PDK1 homologue from the moss Physcomitrella patens. Plant Physiology **158:** 1018-1033. Copyright 2012 © American Society of Plant Biologists. (2) **Dittrich ACN, Devarenne TP** (2012) Perspectives in PDK1 evolution: insights from photosynthetic and non-photosynthetic organisms. Plant Signaling and Behavior, in press. Copyright 2012 © Landes Bioscience.

significance of their discoveries, perhaps because they worked with an artificial substrate and a complex protein extract rather than a purified enzyme. Because tumors and other tissues have high rates of phosphoprotein turnover (Kennedy and Smith, 1954), the authors suspected phosphoproteins were important, but because casein lacks enzymatic activity they did not understand why it would be phosphorylated.

1.1b Protein kinases involved in glycogen metabolism: 1950s – 1960s

Edwin Krebs and Edmond Fischer soon connected protein kinases with the modulation of cellular metabolism, a discovery that ultimately earned the 1992 Nobel Prize in Physiology or Medicine. Much of the pioneering work in protein phosphorylation and dephosphorylation involved enzymes of glycogen metabolism. Krebs and Fischer initially reported that glycogen phosphorylase, the enzyme that catalyzes the rate-limiting step of glycogen breakdown, is converted from its inactive form (phosphorylase b) to its active form (phosphorylase a) in an ATP- and divalent cation-dependent manner (Fischer and Krebs, 1955). Subsequent experiments showed this conversion is due to the activity of an enzyme they named phosphorylase kinase (Krebs and Fischer, 1956; Krebs et al., 1958). Phosphorylase kinase phosphorylates phosphorylase b, which consists of 2 identical subunits (Krebs et al., 1958), at a single serine residue per subunit to stimulate phosphorylase b dimerization to the active phosphorylase a (Fischer et al., 1959). About a decade later, cAMP-dependent protein kinase (also called protein kinase A, or PKA) was found to perform dual functions, both stimulating glycogen breakdown through an activating phosphorylation of phosphorylase kinase (Walsh et al., 1968) and inhibiting glycogen synthesis through an

inhibitory phosphorylation of glycogen synthase (Soderling et al., 1970). Much later, an enzyme that could convert active phosphorylase a back to inactive phosphorylase b by dephosphorylating it was discovered (Ingebritsen and Cohen, 1983).

1.1c Key insights into the nature of protein kinases: 1970s – 1990s

Before 1970 almost all reports of protein phosphorylation were confined to the enzymes of glycogen metabolism, so for many years the ubiquitous nature of protein phosphorylation was not appreciated. However, numerous phosphorylated proteins from different systems were reported throughout the 1970s and 1980s (Krebs and Beavo, 1979). Many important advances were made in the study of protein phosphorylation during these decades.

The discovery of a natural protein kinase substrate without enzymatic activity, histone H1 which is phosphorylated in response to hormone stimulation (Langan, 1969), first suggested that protein kinases act on a wide range of targets in vivo. Early reports of enzymes that were phosphorylated at multiple amino acids (Soderling, 1979) hinted at the phenomenon of multisite phosphorylation, which is now established as a common occurrence in many kinase substrates (Cohen, 2000). In 1980 the identification of two tyrosine kinases, the membrane-associated epidermal growth factor receptor (EGFR) (Ushiro and Cohen, 1980) and the cytoplasmic kinase Src (Hunter and Sefton, 1980), led to the first realization that amino acids other than serine and threonine could be phosphorylated; we now know that histidine and aspartate (Saito, 2001; Besant and Attwood, 2005), and possibly arginine and lysine (Besant et al., 2009), can also be targeted by eukaryotic kinases and phosphatases.

Particularly important was elucidation of the PKA catalytic subunit sequence (Shoji et al., 1981), which enabled other scientists to understand that the catalytic domains of protein kinases share a great deal of sequence similarity (Barker and Dayhoff, 1982; Housey et al., 1987). Because of these and other studies it became possible to recombinantly express (Slice and Taylor, 1989) and determine the crystal structure of the PKA catalytic subunit (Knighton et al., 1991a; Knighton et al., 1991b). The relative simplicity of PKA made it a model representative of the protein kinase family. The PKA structure was so important because all eukaryotic protein kinase catalytic domains are broadly similar, comprising a smaller N-terminal lobe, a larger C-terminal lobe, and an ATP-binding active site between the lobes (Dissmeyer and Schnittger, 2011).

1.1d High throughput analysis of protein phosphorylation: 2000s

The contributions of early experiments to our understanding of protein phosphorylation can hardly be overstated. However, the enormous number of proteins regulated by kinases and phosphatases makes it difficult to form an accurate picture of protein phosphorylation by studying individual proteins. Technological advances of recent years have now made it feasible to investigate protein phosphorylation at a systems level. The rapid progress that has been made is impressive.

Experimental schemes for the high-throughput detection of phosphorylation sites via mass spectrometry were in the early stages of development just over a decade ago (Zhou et al., 2001; Oda et al., 2001); now, the results of multiple studies can be analyzed in parallel to more accurately represent an organism's phosphoproteome (Yachie et al.,

2011; Amoutzias et al., 2012). The development of protein (Zhu et al., 2000; Ptacek et al., 2005) and peptide (Houseman et al., 2002) chips has also greatly facilitated the analysis of kinase activities. Finally, methods for quantitative comparison of changes in the phosphoproteome have been developed recently, usually utilizing differential sample labeling followed by mass spectrometric analysis (Derouiche et al., 2011; Kosako and Nagano, 2011). These innovative techniques are exponentially increasing our understanding of the intricacies of protein kinase networks and global protein phosphorylation status in many organisms.

1.2 Protein phosphorylation as a ubiquitous regulatory mechanism

A vast body of evidence, including the few notable cases discussed above, has established beyond a doubt that reversible protein phosphorylation is an exceedingly common and important mechanism by which cells control the activity, localization, stability, and interactions of thousands of proteins involved in almost all facets of life. Diverse protein kinases have been found in many archaeal and bacterial genomes in relatively limited numbers (Tyagi et al., 2010), but in eukaryotes the protein kinase superfamily has dramatically expanded to comprise approximately 1.5-4% of all genes in most organisms (Manning et al., 2002). For example, the human genome contains at least 500 protein kinases (Kostich et al., 2002; Manning et al., 2002b), while *Arabidopsis thaliana* is predicted to have over 1000 (Wang et al., 2003). Many, though not all, of these kinases participate in signal transduction cascades, which are typically initiated by external stimuli and proceed through several intermediate protein phosphorylation events that culminate in the modulation of cellular programs.

1.2a Protein kinase catalytic mechanism

Protein kinases function by transferring the terminal (or γ) phosphate from ATP to a Ser, Thr, Tyr, or His (Dissmeyer and Schnittger, 2011) residue within the substrate protein (Fig. 1; Fig. 2A). In the prototypical kinase PKA, catalysis is accomplished by several key amino acids within the active site: Lys72, Glu 91, Asp166, Lys168, Asn171, and Asp184 (Johnson et al., 1996; Adams, 2001), which are conserved in other protein kinases. The side chains of these amino acids interact with ATP, a divalent cation (in this case Mg^{2+}, but some kinases prefer Mn^{2+}), and the hydroxyl group of the substrate amino acid (a Ser in the case of PKA) that will be phosphorylated. Together, they facilitate transfer of the γ phosphate to the substrate, with Asp166 acting as a base to accept a proton from the substrate Ser and enabling its hydroxyl group to undergo nucleophilic attack on the γ phosphate (Johnson et al., 1996; Adams, 2001; Fig. 2B).

1.2b Modulation of protein function by phosphorylation

Phosphorylation adds a bulky group with two negative charges (Fig. 2A), and thus can alter protein function in several ways (Dissmeyer and Schnittger, 2011). The charge and steric properties of the phosphate group often induce conformational changes (Fig. 3A), as occurs in numerous protein kinases after phosphorylation of the activation loop, and may activate (Fig. 3B) or inactivate (Fig. 3C) the protein. A second possible outcome of phosphorylation is alteration of protein-protein interactions. Phosphorylation may promote new protein-protein interactions (Fig. 3D), such as with 14-3-3 proteins that specifically recognize phosphorylated amino acids, or it may inhibit interactions with proteins that can bind the unphosphorylated protein (Fig. 3E). This can indirectly

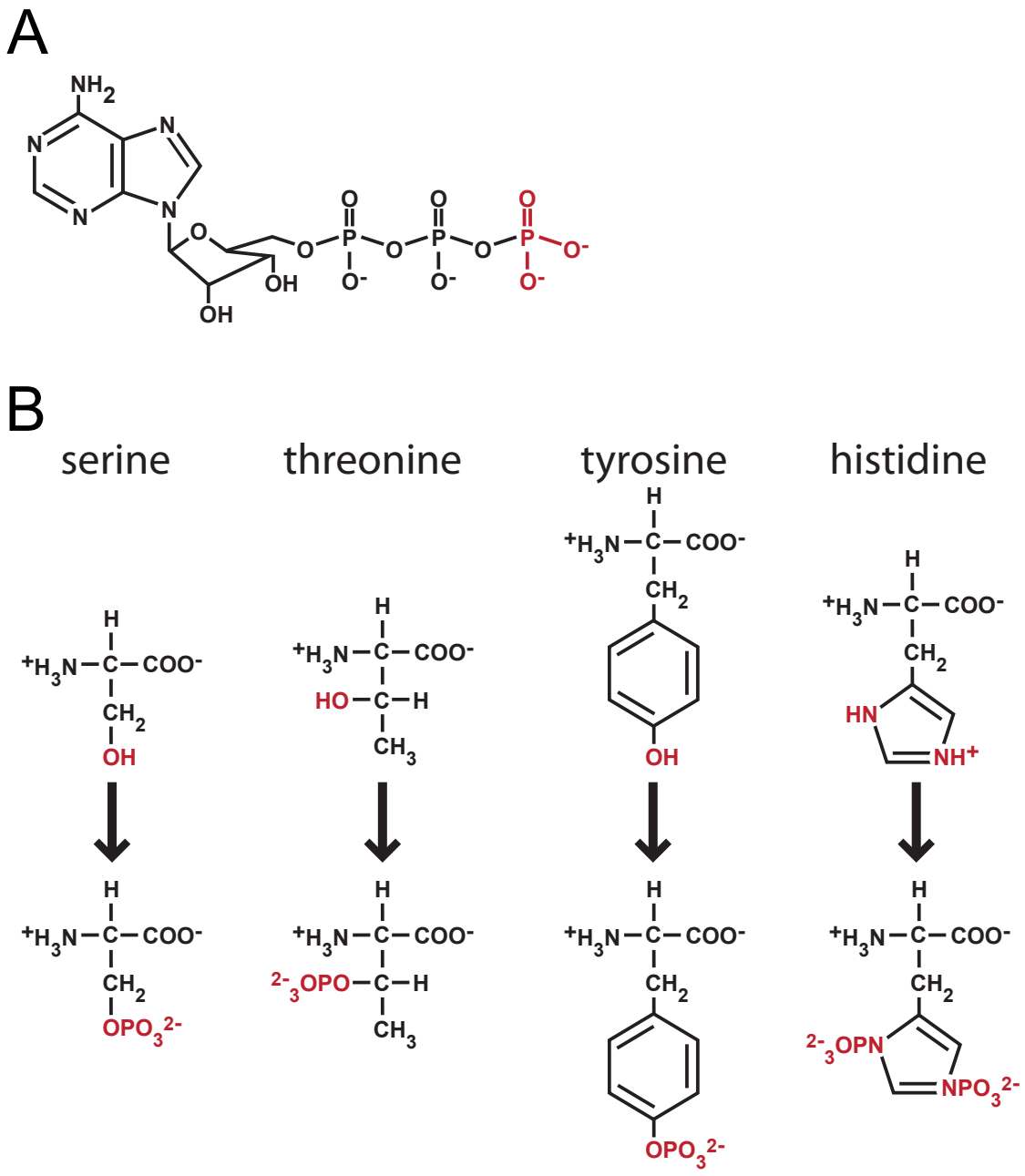


Figure 1. Structure of ATP and commonly phosphorylated amino acids. A, ATP structure with the gamma phosphate indicated in red text. B, The four most commonly phosphorylated amino acids in eukaryotes are shown, with the phosphorylated positions within each amino acid indicated in red text.

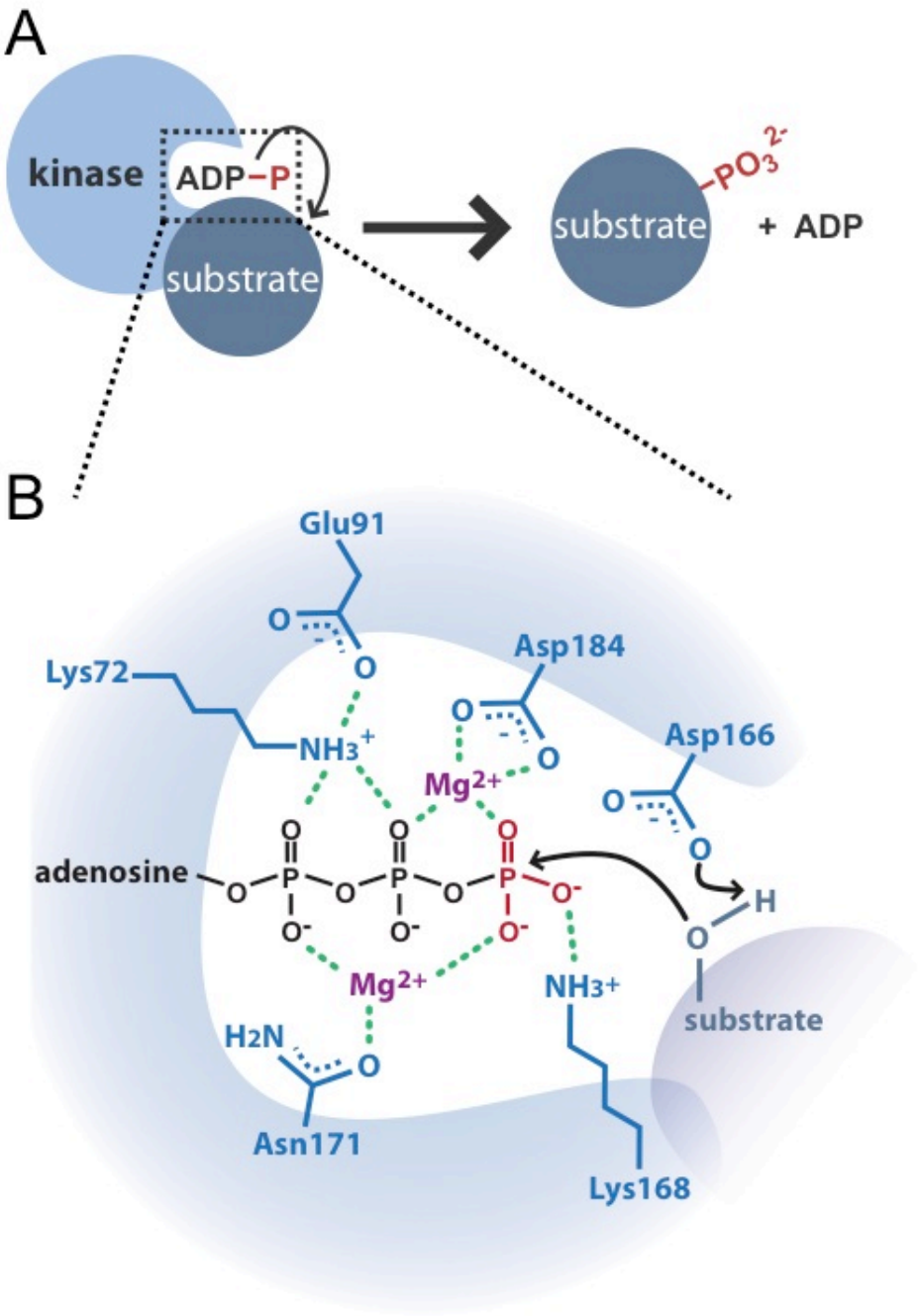


Figure 2. Protein kinase reaction mechanism. A, Diagram of a protein kinase phosphorylating its substrate, showing transfer of the gamma phosphate from ATP to the substrate. The active site within the box is shown in (B) in more detail. B, Detail of the boxed region from (A), showing the general kinase mechanism. Key active site residues are numbered as they are found in the sequence of PKA. Some important contacts are indicated with green dashed lines. This portion of the figure is redrawn using information from Johnson et al., 1996 and Adams, 2001.

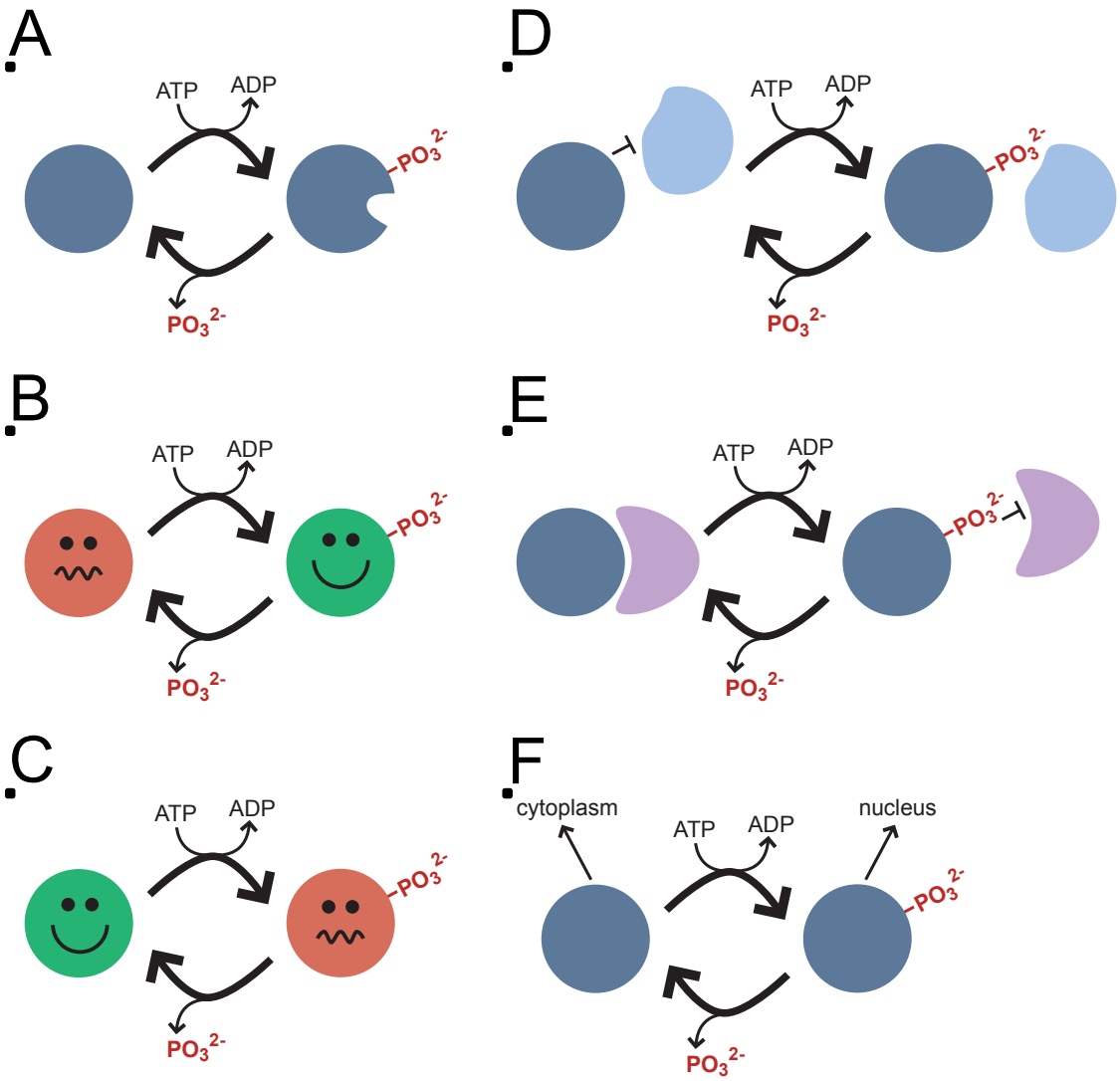


Figure 3. Possible alterations to protein function as a result of phosphorylation. These changes are often interrelated, for example a conformational change might result in protein activation or a new binding partner might change protein stability or localization. A, Phosphorylation may cause conformational changes in the protein. B, Phosphorylation may activate the protein. C, Phosphorylation may inactivate the protein. D, Phosphorylation may enable binding to other interaction partners. E, Phosphorylation may inhibit interaction with partners. F, Phosphrylation may alter protein localization (for example, cytoplasmic/nuclear localization).

influence the stability of some proteins due to crosstalk with ubiquitination enzymes (Hunter, 2007). Finally, subcellular localization may be altered by phosphorylation (Fig. 3F), as occurs with many transcription factors that move from the cytoplasm to the nucleus upon phosphorylation. These functional changes are often related to each other, as illustrated by the mammalian transcription factor p53, a tumor suppressor whose regulation is elaborate and involves several post-translational modifications (Brooks and Gu, 2003). Phosphorylation of p53 occurs in response to DNA damage, and this prevents ubiquitin-mediated proteasomal degradation of p53 by interfering with the E3 ubiquitin ligase's ability to access p53. Thus, p53 is stabilized within the cell and is able to interact with other binding partners. One interactor is a prolyl isomerase that binds phosphorylated p53 and helps activate its transcriptional activity in the nucleus (Brooks and Gu, 2003).

1.3 Classification of eukaryotic protein kinases

1.3a Groups of protein kinases

Early efforts to classify eukaryotic protein kinases utilized catalytic domain comparisons to identify four major kinase groups: AGC (named for protein kinases A, G, and C), CaMK (calcium/calmodulin-dependent kinases), CMGC (named for cyclin−dependent kinases, mitogen−activated protein kinases, glycogen synthase kinases, and CDK−like kinases), PTK (protein tyrosine kinases), plus 1 group of relatively unrelated "other" kinases (Hanks and Hunter, 1995). Later, four additional groups were identified: CK1 (named for casein kinase 1), RGC (receptor guanylate cyclases), STE (named for the yeast Sterile kinases), and TKL (tyrosine kinase-like kinases) (Dittmeyer and

Schnittger, 2011). Catalytic domain sequences are still used to place protein kinases within these groups, and the majority of protein kinases fall into one of the eight "typical" protein kinase categories mentioned above, though a number of highly divergent ("atypical") and catalytically inactive kinases also exist.

1.3b Protein kinase conservation across species

It has become apparent that the protein kinase superfamily is of ancient origin, and that only a few kinases are universally required for eukaryotic life. Phylogenetic analysis of the kinomes of several highly divergent species suggests that kinases have undergone many lineage-specific expansions and reductions (Manning et al., 2002a; Goldberg et al., 2006; Manning et al., 2011).

Some members of the AGC, CaMK, CMGC, and PTK groups are found in budding yeast (*Saccharomyces cerevisiae*), nematode (*Caenorhabditis elegans*), and fruit fly (*Drosophila melanogaster*) (Manning et al., 2002a). Even before the *A. thaliana* genome was sequenced, plant protein kinases belonging to the AGC, CaMK, and CMGC groups were evident (Stone and Walker, 1995; Hardie, 1999); more recent analyses have reported additional conserved kinases in both *A. thaliana* (Wang et al., 2003) and rice (Ding et al., 2009). However, the majority of protein kinases, including many AGC, CaMK, and CMGC kinases, are not conserved in diverse organisms. Only about 40-60 protein kinases are found in all eukaryotes examined, suggesting that they were present in the common eukaryotic ancestor (Manning et al., 2002a; Goldberg et al., 2006; Manning et al., 2011).

1.4 Conserved characteristics of protein kinases

Almost all eukaryotic protein kinases have a relatively conserved catalytic domain of approximately 260-300 amino acids. The first protein kinase classification schemes divided the catalytic domain into 12 distinct sub-domains, I through XI, each with its own features. Similarities and differences within each sub-domain were used to organize protein kinases within groups (Hanks et al., 1988; Hanks and Hunter, 1995). This classification system has persisted, and several conserved sequence (Stone and Walker, 1995; Hanks, 2003) and structural (Biondi et al., 2002; Scheef and Bourne, 2005) characteristics have emerged through the analysis of many protein kinases.

1.4a Conserved catalytic domain sequences and structures

Two important active site residues (corresponding to Lys72 and Glu91 of PKA) reside within the smaller N-terminal lobe of the kinase domain (Fig. 2; Fig. 4A), which is primarily composed of β-strands (Fig. 4B). The residue corresponding to Lys72 of PKA is found within the β3 strand of subdomain II, and the residue corresponding to Glu91 of PKA is found within the αC helix of subdomain III, which undergoes important structural changes after activation loop phosphorylation. In addition, the N-terminal lobe has a glycine-rich loop located in the β1 strand of subdomain I. The backbone atoms of this loop interact with the α and β phosphates of ATP and help position the γ phosphate for transfer to a kinase substrate (Dittmeyer and Schnittger, 2011). The larger C-terminal lobe of the kinase domain is primarily composed of α-helices (Fig. 4B) and contains four key active site residues (corresponding to Asp166, Lys168, Asn171, and Asp184 of PKA) (Fig. 2; Fig. 4A). The DxKxxN motif,

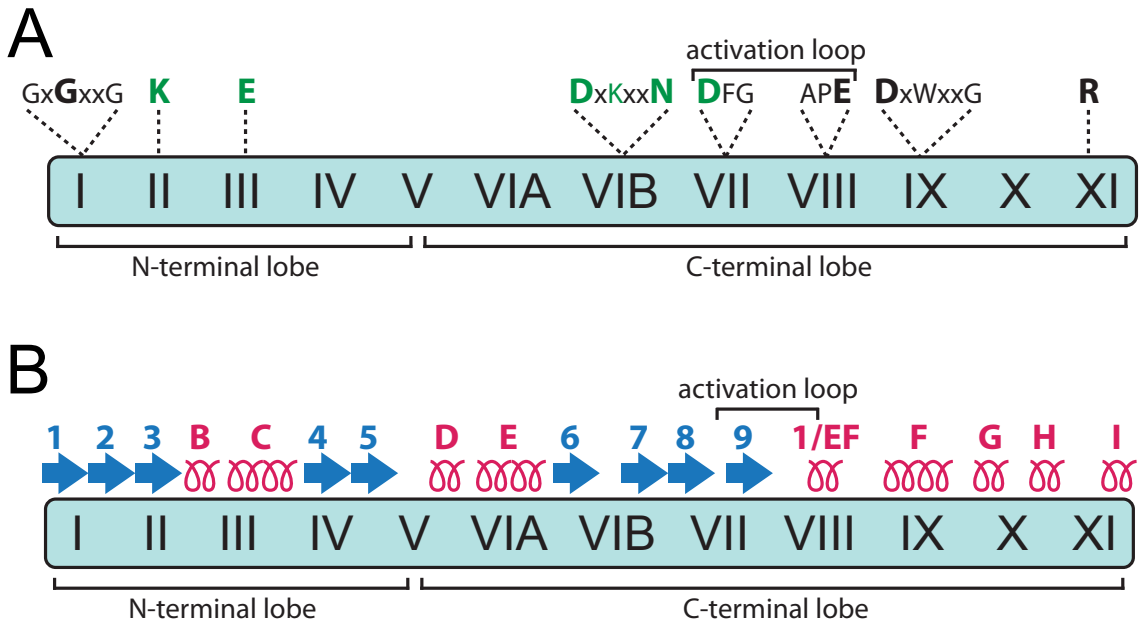


Figure 4. Conserved sequence and secondary structural features of protein kinase catalytic domains. Diagrams and elements are not drawn to scale, but the approximate position of each element within the catalytic domain (light blue rectangle) is indicated. Subdomains are indicated with Roman numerals inside the catalytic domain, the N- and C-terminal lobes are indicated below it, and the activation loop (for kinases that have one) is indicated above it. A, Conserved sequence elements of protein kinases. The subdomain where each sequence may be found is indicated with dashed lines. The one-letter amino acid code is used, with x used to represent any amino acid. Amino acids in green are those found in the active site of PKA in Figure 2, and amino acids shown in bold text are invariant in protein kinases. Information in this diagram was taken from Stone and Walker, 1995 and Hanks, 2003. B, Conserved secondary structural elements of protein kinases. Blue arrows represent β-strands (numbered 1-9), and pink spirals represent α-helices (numbered B-I). Information in this diagram was taken from Biondi et al., 2002 and Scheef and Bourne, 2005.

corresponding to the catalytic Asp166 plus Lys168 and Asn171 of PKA (Fig. 2), is located between the β6 and β7 strands in subdomain VIB. The activation loop, which contains a conserved phosphorylation site in many protein kinases, spans subdomains VII and VIII, beginning at the conserved DFG motif and ending at the APE motif. This loop is mostly unstructured, only containing the β9 strand and part of the αEF helix (also called the α1 helix; Scheeff and Bourne, 2005). Finally, the DxWxxG motif and a conserved Arg are located within subdomains IX and XI, respectively. The DxWxxG motif is located within the αF helix, while the conserved Arg is found in an unstructured region between the αH and αI helices.

1.4b Alterations to conserved structures during activation

Together the conserved sequence and secondary structural elements perform important functions for protein kinases. As mentioned previously, at least six conserved amino acids (green in Fig. 4) participate in active-site interactions with ATP, optimally positioning it for γ phosphate transfer. In particular, changes to the activation loop and the αC helix with its conserved Glu are critical determinants of whether a protein kinase is active or inactive (Johnson et al., 1996). If the activation loop is unphosphorylated, the kinase is typically found in an inactive, open conformation in which the αC helix is disordered, the DFG motif partially occupies the active site, and therefore the active site cannot accommodate and position an ATP molecule. Upon activation loop phosphorylation, the kinase adopts an active, closed conformation in which the αC helix is stabilized and active site residues are correctly oriented to facilitate ATP hydrolysis (Johnson et al., 1996; Biondi et al., 2002; Biondi, 2004).

1.5 Overview of AGC protein kinases and general mechanisms of activation

1.5a General AGC kinase functions

The AGC kinase group, which was named for three of its representatives, PKA, cGMP-dependent protein kinase (PKG), and protein kinase C (PKC), comprises 60 members in human (Manning, 2002; Pearce et al., 2010) and at least 39 members in *A. thaliana* (Bögre et al., 2003). Interestingly, functions have not yet been ascribed to many AGC kinases; the majority of research has focused on AGC kinases with obvious roles in human disease, plus a few plant AGC kinases with widely varied functions (Pearce et al., 2010; Bögre et al., 2003). The AGC kinases that have been investigated are known to regulate a wide range of essential cellular processes, including metabolism, transcription and translation, cell growth and proliferation, apoptosis, and hormone and insulin response in animals (Pearce et al., 2010) and plants alike (Bögre et al., 2003; Galvan-Ampudia and Offringa, 2007; Zhang and McCormick, 2009). Many of these AGC kinases have remarkably similar mechanisms of activation, though future research will doubtlessly show that many features of AGC kinases are not generalizable.

1.5b Regulation of mammalian AGC kinase activity

Three main conserved phosphorylation sites (Pearce et al., 2010; Bayascas, 2010), one of which was characterized recently (Hauge et al., 2007), regulate the activity of several important mammalian AGC kinases. These phosphorylation sites are depicted in Figure 5.

The first phosphorylation site is located within a turn motif typically found near the C-terminus of the AGC kinase. Because this site was only investigated in detail recently

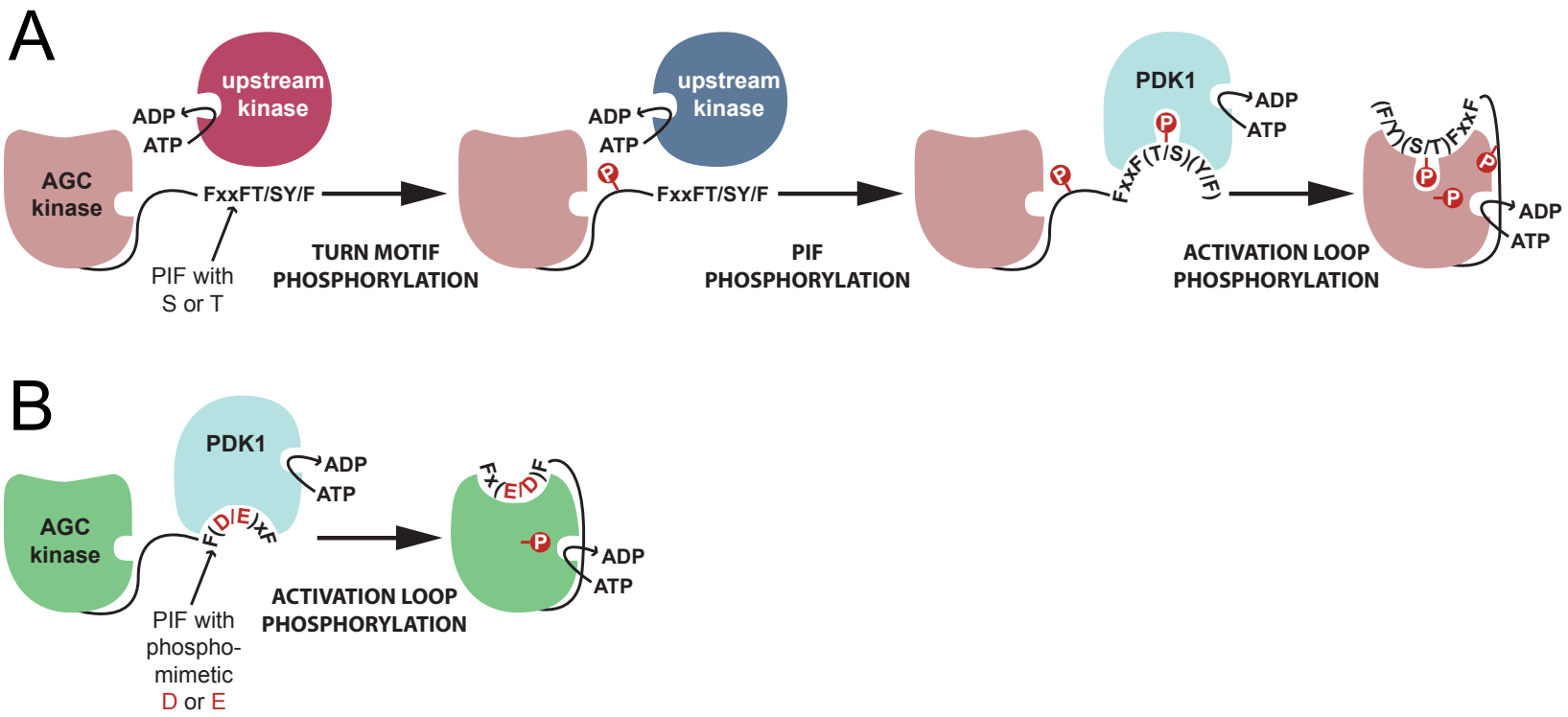


Figure 5. General schemes for activating several AGC kinases in mammals and plants. A, A number of mammalian AGC kinases are regulated by phosphorylation at 3 sites. The first site is located in the turn motif, usually near the C-terminus of the AGC kinase, and its phosphorylation stabilizes the kinase. The second site is within the hydrophobic PDK1-interacting fragment (PIF motif), typically at the C-terminus, and phosphorylation is usually required for interaction with 3-phosphoinositide-dependent protein kinase 1 (PDK1). The third site is within the activation loop. The kinases that phosphorylate turn and PIF sites can vary, but PDK1 is usually responsible for activation loop phosphorylation. Fully phosphorylated AGC kinases have maximal stability and activity. B, Most plant AGC kinases lack phosphorylation sites corresponding to the turn and PIF motif sites in mammalian AGC kinases. Instead, an Asp or Glu is often found in the PIF. PDK1 is able to interact with the PIF motif and phosphorylate AGC kinases within the activation loop. Again, PDK1 phosphorylation confers full activity to plant AGC kinases.

(Hauge et al., 2007), less is known about it than the other two phosphorylation sites. In some AGC kinases (PKB, S6K, some isoforms of PKC) upstream kinases like the TOR (target of rapamycin) kinase are capable of phosphorylating the turn motif, whereas in other cases the turn motif is subject to autophosphorylation (PKA, other isoforms of PKC) or phosphorylation by a different upstream kinase (Hauge et al., 2007; Alessi et al., 2009). In any case, the ultimate result is the same. Turn motif phosphorylation is important for increasing the stability of the AGC kinase and protecting the hydrophobic motif (discussed in the following paragraph) from dephosphorylation. The phosphorylated turn motif site also acts as a "zipper" to bring the hydrophobic motif in close proximity to its binding pocket within the kinase (Fig. 5A). This is thought to increase AGC kinase activity (Hauge et al., 2007). In some AGC kinases the turn motif is constitutively phosphorylated, but in others it only occurs after stimulation by extracellular agonists like growth factors or hormones (Hauge et al., 2007).

The second phosphorylation site is located in a hydrophobic motif typically found at the C-terminus of the AGC kinase, approximately 20 amino acids downstream of the turn motif site. The hydrophobic motif consists of three aromatic amino acids surrounding a Ser or Thr phosphorylation site; its consensus sequence is FxxF(S/T)(F/Y) (Frödin et al., 2002). This site is typically subject to either autophosphorylation or phosphorylation by the TOR (Target of Rapamycin)-containing protein kinase complexes TORC1 or TORC2 (Sarbassov et al., 2005; Alessi et al., 2009; Bayascas, 2010). In some mammalian and most plant AGC kinases, the phosphorylatable Ser or Thr is missing; instead an Asp or Glu mimics a phosphorylated amino acid (Bayascas,

2010; Bögre et al., 2003; Fig. 5B). Phosphorylation of the hydrophobic motif (or the presence of a phosphomimetic amino acid) promotes its association with a complementary pocket within 3-phosphoinositide-dependent protein kinase 1 (PDK1), which is also an AGC kinase. Accordingly, the hydrophobic motif is also termed the PDK1-interacting fragment (PIF) and the corresponding pocket within PDK1 is the PIF-binding pocket (Fig. 5). Interaction with, and subsequent phosphorylation by, PDK1 is required for full activity of most of the human AGC kinases that are substrates of PDK1 (Mora et al., 2004; Bayascas et al., 2008; Bayascas et al., 2010; Pearce et al., 2010).

The final phosphorylation site is located in the activation loop, also called the T-loop (Bögre et al., 2003). As mentioned previously, the activation loop extends from the conserved sequence motifs DFG (in subdomain VII) to APE (in subdomain VIII) of the catalytic domain of many kinases (Fig. 4), and it contains a highly conserved Ser or Thr phosphorylation site (Hanks and Hunter, 1995; Johnson et al., 1996). PDK1 has a relatively large number of AGC kinase substrates (Pearce et al., 2010; Zegzouti et al., 2006b), which will be discussed in more detail in later sections. For most of these PDK1 substrates, activation loop phosphorylation is crucial for achieving maximum kinase activity, both in mammals (Johnson et al., 1996; Mora et al., 2004; Bayascas et al., 2008; Bayascas et al., 2010; Pearce et al., 2010) and in plants (Bögre et al., 2003; Anthony et al., 2004; Anthony et al., 2006; Devarenne et al., 2006; Zegzouti et al., 2006a; Zegzouti et al., 2006b). Activation loop phosphorylation by PDK1 typically occurs only after docking of the AGC kinase substrate's hydrophobic motif within the PIF-binding pocket located in the small lobe of the PDK1 kinase domain (Biondi et al., 2002; Fig. 5). Many

substrate AGC kinases also contain a PIF-binding pocket for binding their own PIF, which stabilizes the fully kinase-active conformation following PDK1 phosphorylation (Frödin et al., 2002; Biondi, 2004; Fig. 5). For these reasons, PDK1 is a critical point at which AGC kinase signaling is regulated. Because several AGC kinases are misregulated in a variety of diseases including cancer, PDK1 and its interactions with substrates have been intensively studied in mammalian systems in pursuit of treatment strategies (Mora et al., 2004; Pearce et al., 2010).

1.5c Regulation of plant AGC kinase activity

Interestingly, only the activation loop phosphorylation site appears to be broadly conserved in plant AGC kinases (Fig. 5B). A few kinases such as S6K, IRE, and NDR have a phosphorylatable Ser or Thr within their PIF motif (Bögre et al., 2003), but the significance of this is largely unknown. Tmajority of plant PDK1 substrates have a phosphomimetic Asp or Glu in the PIF motif (Fig. 5B). As in mammals, PDK1 docks with the PIF using its PIF-binding pocket, then phosphorylates its substrates within the activation loop to increase their activity (Bögre et al., 2003; Zegzouti et al., 2006b; Fig. 5B). Unfortunately, the plant AGC kinases have been much less studied than their mammalian counterparts. Consequently, fewer details are known about plant PDK1-substrate interactions and plant AGC kinase activation.

1.6 Roles of PDK1 in animals and yeast

In the years since its identification, PDK1 has been demonstrated to phosphorylate more than 20 of the 60 human AGC kinases, thereby coordinating numerous and diverse cellular processes, including cell survival and apoptosis, cell growth and division,

hormone responses, and protein synthesis (Mora et al., 2004; Pearce et al., 2010). Evidently, the ability of PDK1 to activate a large array of AGC kinases is a crucial regulatory mechanism for many important signaling pathways.

1.6a Discovery of PDK1

PDK1 was initially identified by chromatographic fractionation of muscle and brain protein extracts as the upstream kinase responsible for phosphorylating the activation loop of protein kinase B (PKB, also called Akt; Alessi et al., 1997b; Stokoe et al., 1997). PKB is a highly studied AGC kinase that is activated upon perception of second messengers like insulin and growth factors. PKB activation occurs upon phosphoinositide 3-kinase-mediated production of the signaling lipid PtdIns(3,4,5)P_3 (Vanhaesebroeck et al., 2012). PtdIns(3,4,5)P_3 then directs colocalization of PDK1 and PKB at the plasma membrane and promotes PDK1-PKB interaction (Borgatti et al., 2003; Calleja et al., 2007). PDK1-phosphorylated PKB has 30-fold higher activity than wild type protein, and activation of PKB ultimately enables it to promote cell survival (Alessi et al., 1997b). PDK1's roles apart from PKB became an important focus of research efforts when it became apparent that a host of substrates contained a conserved PDK1 phosphorylation motif (Pearce et al., 2010; Kobayashi and Cohen, 1999; Le Good et al., 1998; Pullen et al., 1998).

Though not present in prokaryotes, *PDK1*-like sequences have been identified in all eukaryotes studied so far including mice (Dong et al., 1999), nematodes (Paradis et al., 1999), yeast (Casamayor et al., 1999; Niederberger and Schweingruber, 1999), slime molds (Kamimura and Devreotes, 2010), insects (Alessi et al., 1997a; Cho et al., 2001),

plants (Deak et al., 1999; Devarenne et al., 2006; Matsui et al., 2010; Dittrich and Devarenne, 2012), multicellular fungi, and gastropods (Silber et al., 2004). As might be expected because of its role as a key regulator of AGC kinase signaling, loss of *PDK1* is lethal in yeast (Casamayor et al., 1999; Niederberger and Schweingruber, 1999), *D. melanogaster* (Rintelen et al., 2001), and mice (Lawlor et al., 2002).

1.6b Mechanism of PDK1-PKB interaction

Cloning of the human and fruit fly PDK1 genes (Alessi et al., 1997a) led to an initial understanding of how PDK1 could interact with PKB. In addition to its catalytic domain, PDK1 has a pleckstrin homology (PH) domain that enables high-affinity interactions with several phospholipids, especially phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) and phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3) (Fig. 6A). The most well established lipid regulators of mammalian PDK1 are PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, which are produced by class I phosphatidylinositol 3-kinases (PI3Ks) in response to growth factor or insulin perception and modulate the cellular environment through downstream signaling pathways like PDK1-PKB (Vanhaesebroeck et al., 2012). Both PDK1 and PKB bind PtdIns(3,4,5)P_3 through their PH domains. Structural and functional studies on lipid-free and PtdIns(3,4,5)P_3-bound PDK1 PH domains have identified the residues that contribute to lipid binding and shown that, unlike for PKB, lipid binding does not induce a conformational change in the PDK1 PH domain (Komander et al., 2004; Millburn et al., 2003).

Though lipid binding is now thought to facilitate PDK1 and PKB colocalization at the plasma membrane (Pearce et al., 2010; Mora et al., 2004), it has been difficult to

conclusively determine whether PtdIns(3,4,5)P_3 alters the localization of PDK1. A recent report has found that the abundant and constitutively present lipid phosphatidylserine recruits mammalian PDK1 to the plasma membrane via a small pocket within the PH domain that specifically mediates PS binding (Lucas and Cho, 2011). This result provides one possible mechanism for the earlier finding that a substantial fraction of PDK1 is membrane-bound even when PtdIns(3,4,5)P_3 production has not been stimulated (Currie et al., 1999), and in the future it will be interesting to test whether other PDK1s are also recruited to membranes through interactions with abundant lipids.

1.6c Differences between mammalian and yeast PDK1

As might be expected from their drastic differences in form and lifestyle, mammalian and yeast PDK1 homologues differ in their structure and function. Most reported PDK1s resemble human PDK1, with a domain architecture of kinase domain + C-terminal PH domain (Fig. 6A). In contrast, the *S. cerevisiae* PDK1 homologues Pkh1 and Pkh2 lack an obvious lipid-binding domain (Casamayor et al., 1999). Unexpectedly, the activity of Pkh1/2 is regulated by sphingoid bases like phytosphingosine (Friant et al., 2001; Fig. 6B), raising the possibility that cryptic lipid-binding domains may exist in these proteins. Nevertheless, both full-length human PDK1 and PDK1 lacking the PH domain can complement loss of Pkh1/2 (Casamayor et al., 1999). This suggests some aspects of PDK1 functionality remain unchanged throughout evolution, particularly its ability to interact with and phosphorylate AGC kinase substrates.

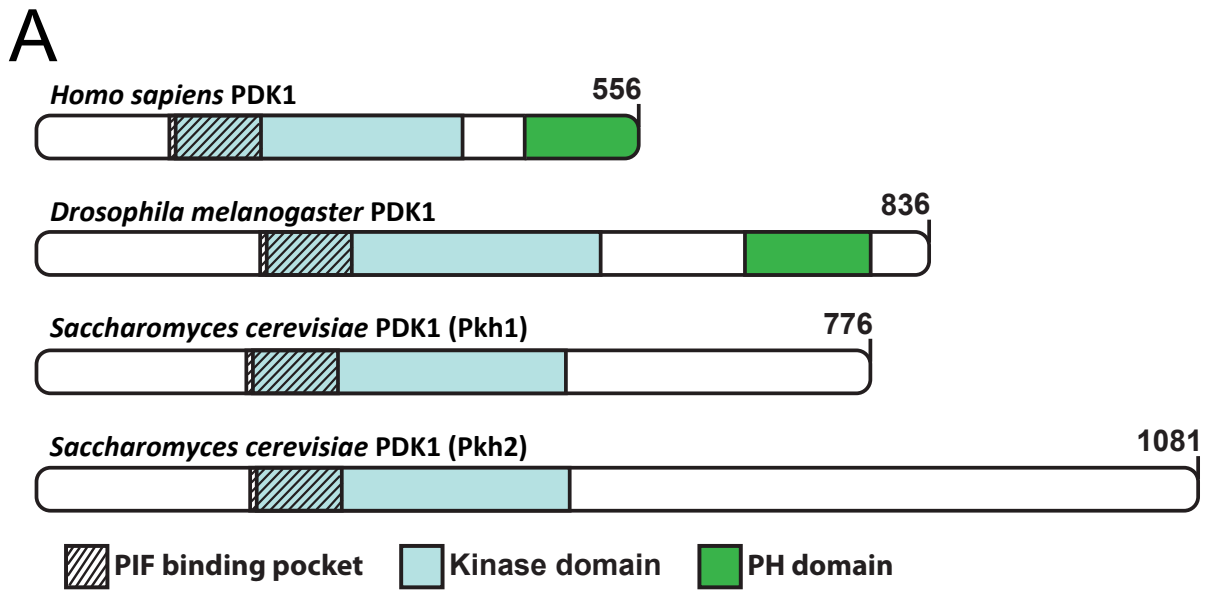
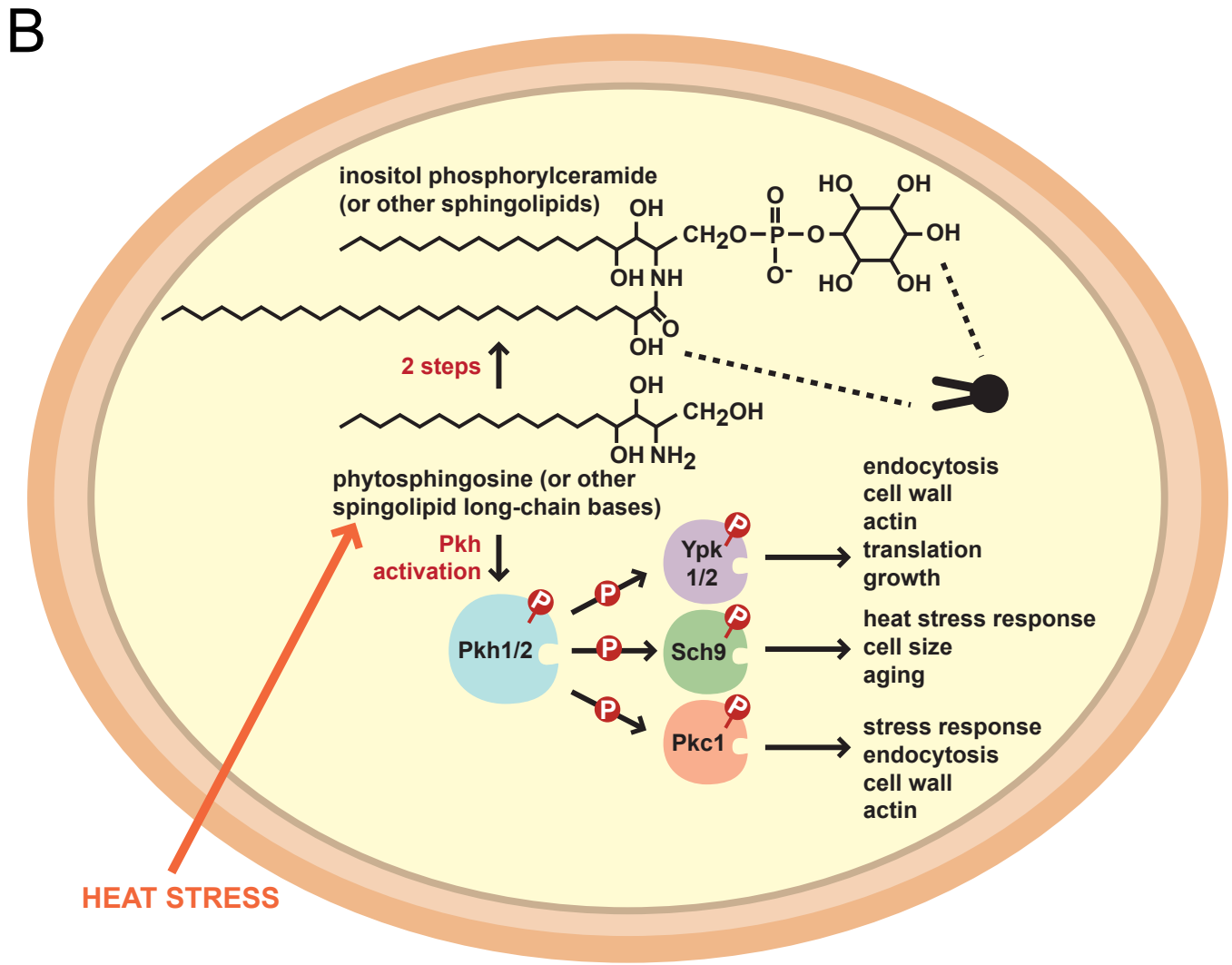


Figure 6. Protein domains and roles of PDK1 from animals and budding yeast. A, Diagrams of PDK1 proteins from human, fruit fly, and budding yeast with the approximate locations of functional domains within each protein. The number of amino acids is indicated at the top right corner of each protein. B, Simplified diagram of PDK1-dependent pathways in budding yeast that are essential for organism survival. Inositol phosphorylceramide is a plasma membrane sphingolipid, but long-chain sphingoid bases are found in the endoplasmic reticulum (Dickson et al., 2006; Dickson et al., 2008). C, Simplified diagram of a few of the many PDK1-dependent pathways in animals, including the approximate human homologues of budding yeast proteins from part (B). Both PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 are plasma membrane phospholipids (Vanhaesebroeck, 2012).



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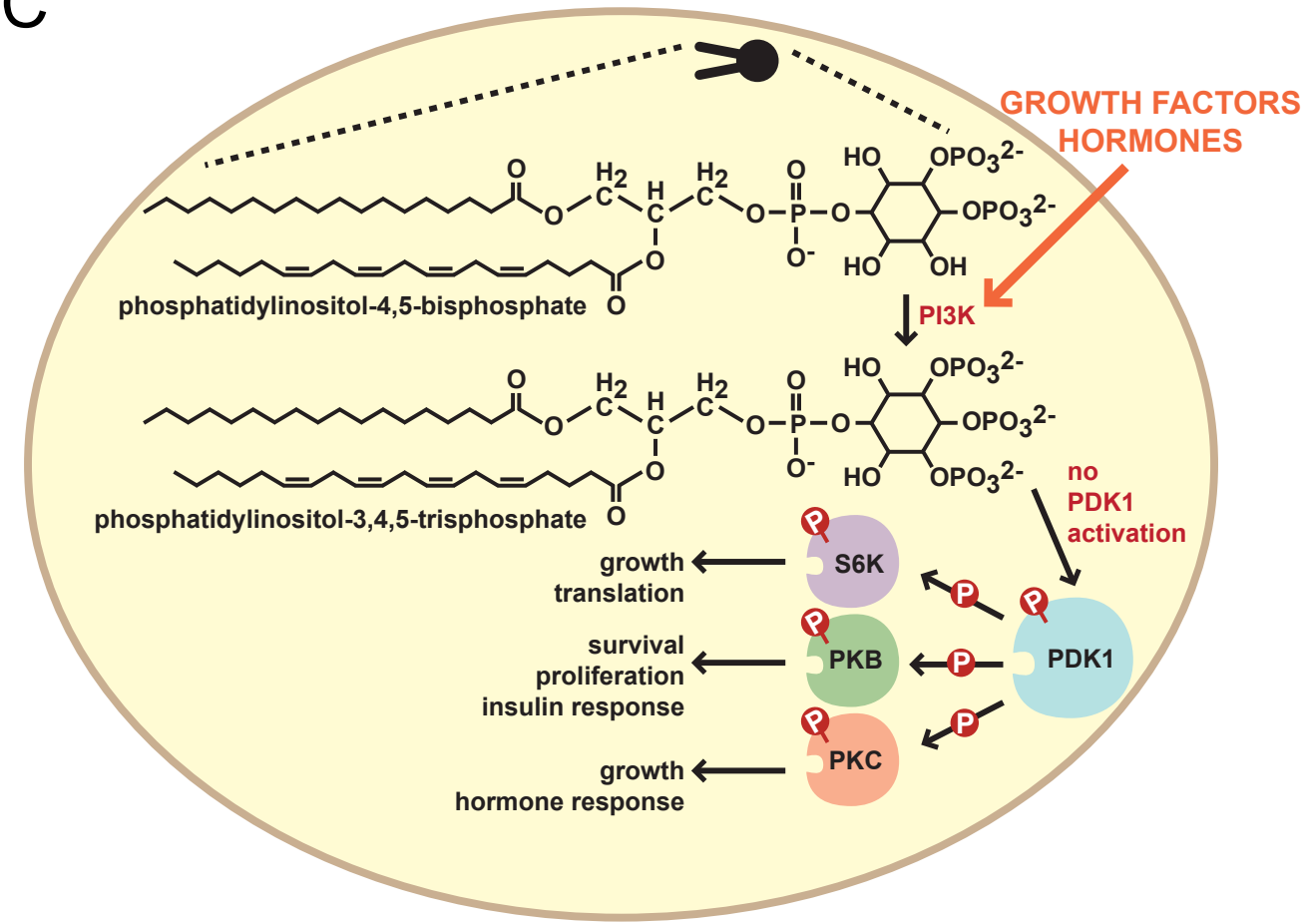

Figure 6, continued.

S. cerevisiae Pkh1/2 activate pathways that regulate endocytosis, growth and stress response, protein translation, actin dynamics, cell lifespan, and ensure proper cell size (Dickson et al., 2006; Dickson, 2008; Fig. 6B). Loss of Pkh1/2 function is thought to be lethal due to cell wall defects that lead to lysis (Inagaki et al., 1999). Interestingly, some functions downstream of Pkh1/2 and mammalian PDK1 appear to be conserved, including translation, cell growth and proliferation, and cell size and lifespan (Fig. 6, B and C). However, loss-of-function phenotypes in *S. cerevisiae* and mammalian cells indicate that some new or divergent functions downstream of PDK1 have evolved since the split between fungi and animals (Fig. 6C). Animal-specific functions controlled by PDK1 include regulation of embryo development (Lawlor et al., 2002), liver, brain, and heart function (Lawlor et al., 2002; Mora et al., 2003), and response to insulin and growth factors (Mora et al., 2004) (Fig. 6C). Unlike Pkh1/2, mammalian PDK1 does not appear to play a role in endocytosis, nor is it involved in cell wall maintenance (since mammalian cells lack a wall).

1.7 Roles of PDK1 in plants

The first plant homologues of PDK1 were reported just two years after initial discovery of the human PDK1 (Deak et al., 1999). Mammalian PDK1 homologues have been highly studied by researchers seeking targets for drugs to treat cancer and other human diseases, but less is known about the functions of PDK1 in plants.

1.7a Conserved and distinct functions of plant PDK1s

Interestingly, in contrast to the other studied *PDK1* genes, *PDK1* from *A. thaliana* (*AtPDK1*) is non-essential for plant survival (Camehl et al., 2011), although

loss of tomato (*Solanum lycopersicum*) PDK1 (*SlPDK1*) is lethal (Devarenne et al., 2006). The reasons for this discrepancy are not yet clear. Despite the very distant relationship between plants and fungi, *PDK1* from *A. thaliana* has been shown to complement loss of the *S. cerevisiae PDK1* homologues *PKH1* and *PKH2* (Deak et al., 1999). These results further support the idea that PDK1 phosphorylation of its substrates is broadly conserved amongst highly divergent organisms, but that not all pathways regulated by PDK1 are the same in these organisms.

1.7b Structure and function of plant PDK1s

Examination of the *A. thaliana* PDK1 sequence reveals that it possesses many of the conserved amino acids that participate in substrate interaction, catalysis, and lipid binding in human PDK1 (Fig. 7). The experimentally confirmed plant PDK1s very closely resemble human PDK1 in their domain architecture, with a kinase domain + C-terminal PH domain (Deak et al., 1999; Devarenne et al., 2006; Fig. 8A). However, the lipid-binding capabilities of plant and animal PDK1s differ. *A. thaliana* PDK1 is capable of somewhat promiscuous lipid binding in vitro, displaying strong interactions with PtdIns3P, PtdIns(3,4)P_2, PtdIns(4,5)P_2, PtdIns(3,4,5)P_3, and phosphatidic acid (PA) (Deak et al., 1999). Despite these strong in vitro interactions, it is unlikely that either PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 play a role in plant PDK1 regulation for two reasons. First, plants lack a class I PI3K and thus cannot produce PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 as mammals do; second, neither of these lipids has been reliably documented in plant cells (Munnik and Testerink, 2009). Plants do generate PtdIns3P, PtdIns(4,5)P_2, and PA (Munnik and Testerink, 2009), so these lipids might participate in PDK1 regulation.

```
AtPDK1-1  ------------------MLA-------MEKEFDSKLVL------------QGNSSNGAN 23
SlPDK1-1  ------------------MLALVGGEGDMEQEFDAKLKI------------QNNS---AN 27
PpPDK1    ----------------------------MAMDGTSPVSP------------EPNQ---SK 17
HsPDK1    MARTTSQLYDAVPIQSSVVLCSCPSPSMVRTQTESSTPPGIPGGSRQGPAMDGTAAEPRP 60

                                        β1        β2        β3    αB
AtPDK1-1  VSRSKSFSFKAPQ-ENFTSHDFEFGKIYGVGSYSKVVRAKKKETGTVYALKIMDKKFITK 82
SlPDK1-1  TQRSKSFAFRAPQ-ENFTIQDFELGKIYGVGSYSKVVRAKKKDTANVYALKIMDKKFITK 86
PpPDK1    PLDPKQLVMRAPQ-MDFTSNDFLFAKLLGLGSYSKVTKAKRKNTGEIYALKIMNKKHIIR 76
HsPDK1    GAGSLQHAQPPPQPRKKRPEDFKFGKILGEGSFSTVVLARELATSREYAIKILEKRHIIK 120

                  αC        β4    β5        αD    αE
AtPDK1-1  ENKTAYVKLERIVLDQLEHPGIIKLYFTFQDTSSLYMALESCEGGELFDQITRKGRLSED 142
SlPDK1-1  ENKTAYVKLERIVLDQLDHPGVVRLFFTFQDTFSLYMALESCEGGELFDQITRKGRLSED 146
PpPDK1    ENKVKFVKMERMILDQLDHPGVVKLCFTFQDVHSLYMGLECCTGGELFEQIRRSKRMSEE 136
HsPDK1    ENKVPYVTRERDVMSRLDHPFFVKLYFTFQDDEKLYFGLSYAKNGELLKYIRKIGSFDET 180

                  αE   β6        β7    β8      β9
AtPDK1-1  EARFYTAEVVDALEYIHSMGLIHRDIKPENLLLTSDGHIKIADFGSVKPMQDSQITVLPN 202
SlPDK1-1  EARFYAAEVVDALEYIHSMGLIHRDIKPENLLLTSDGHIKIADFGSVKPMQDSRITVLPN 206
PpPDK1    DTRFYTAEIVDILEYIHSQGIVHRDLKPENILISAEGNLKLCDFGSAKMFRPLPNGFFQ- 195
HsPDK1    CTRFYTAEIVSALEYLHGKGIIHRDLKPENILLNEDMHIQITDFGTAKVLSPE------- 233

                      αEF                 αF        αG
AtPDK1-1  AASDDKACTFVGTAAYVPPEVLNSSPATFGNDLWALGCTLYQMLSGTSPFKDASEWLIFQ 262
SlPDK1-1  AASDDKACTFVGTAAYVPPEVLNSSPATFGNDLWALGCTLYQMLSGTSPFKDASEWLIFQ 266
PpPDK1    -SEEEDSSAFVGTAEYVSPEVLHGKSASHSVDLWALGCTIYQMLEGRPPFKAATEYLTFQ 254
HsPDK1    -SKQARANSFVGTAQYVSPELLTEKSACKSSDLWALGCIIYQLVAGLPPFRAGNEYLIFQ 292

          αG          αH              αI
AtPDK1-1  RIIARDIKFPNHFSEAARDLIDRLLDTEPSRRP-GAGSEGYVALKRHPFFNGVDWKNLRS 321
SlPDK1-1  RIIARDIRFPNYFSNEARDIIDQLLDVDPSRRP-GAGPDGYASLKNHPFFSGIDWENLRL 325
PpPDK1    KVMARELSIPSHFSPEAKDLVDSLLNLKPNER---LGVQGYDDIKNHPFFKGFDWSRLRK 311
HsPDK1    KIIKLEYDFPEKFFPKARDLVEKLLVLDATKRLGCEEMEGYGPLKAHPFFESVTWENLHQ 352

AtPDK1-1  QTPPKLAPDP--------------------------ASQTASPERDDTHGSPWNLTHIG 354
SlPDK1-1  QTPPRLAMEP--------------------------KAPSTHSSGDEQDPS-WNPSHIG 357
PpPDK1    MATPKLLKDP--------------------------NTESLDEE--------------- 329
HsPDK1    QTPPKLTAYLPAMSEDDEDCYGNYDNLLSQFGCMQVSSSSSSHSLSASDTGLPQRSGSNI 412

AtPDK1-1  DSLATQNEGHSAPPTSSESSGSITRLASIDSFDSRWQQFLEPGESVLMISAVKKLQKITS 414
SlPDK1-1  DGSVRPNDGNGAAASVSEAGNSITRLASIDSFDSKWKQFLDPGESVLMISMVKKLQKLTS 417
PpPDK1    --------------------------------EKWQAGIIDGLDAFVYDV--------- 347
HsPDK1    EQYIHDLDSNSFELDLQFSEDEKRLLLEKQAGGNPWHQFVENN-LILKMGPVDKRKGLFA 471

AtPDK1-1  KKVQLILTNKPKLIYVDPSKLVVKGNIIWSDNSNDLNVVVTSPSHFKICTPKKVLSFEDA 474
SlPDK1-1  KKVQLILTNKPKLIYVDPSKLVIKGNIIWSDNPNDLSIQVTSPSQFKICTPKKVMSFEDA 477
PpPDK1    ------------------------------------------------------------ 
HsPDK1    RRRQLLLTEGPHLYYVDPVNKVLKGEIPWS---QELRPEAKNFKTFFVHTPNRTYYLMDP 528

AtPDK1-1  KQRASVWKKAIETLQNR---------- 491
SlPDK1-1  KNRAQQWKKAIEALQNR---------- 494
PpPDK1    ---------------------------- 
HsPDK1    SGNAHKWCRKIQEVWRQRYQSHPDAAVQ 556
```

Figure 7. Sequence and structural elements of PDK1 from four species. PDK1s from *A. thaliana* (AtPDK1-1), tomato (SlPDK1-1), the moss *Physcomitrella patens* (PpPDK1), and human (HsPDK1) are aligned. The N-terminal and C-terminal lobes of the kinase domain are highlighted in yellow and blue respectively, and the pleckstrin homology domain is highlighted in green. Active site amino acids that may aid in catalysis are indicated in green text, PIF-binding pocket residues from Frodin et al., 2002 are indicated in pink text, activation loop residues are indicated in maroon text, the putative activation loop site is indicated in red text, and putative phosphoinositide-binding residues are indicated in blue text. Putative secondary structural elements are shown on the alignment, as determined in the human PDK1 kinase domain crystal structure (Biondi et al., 2002).

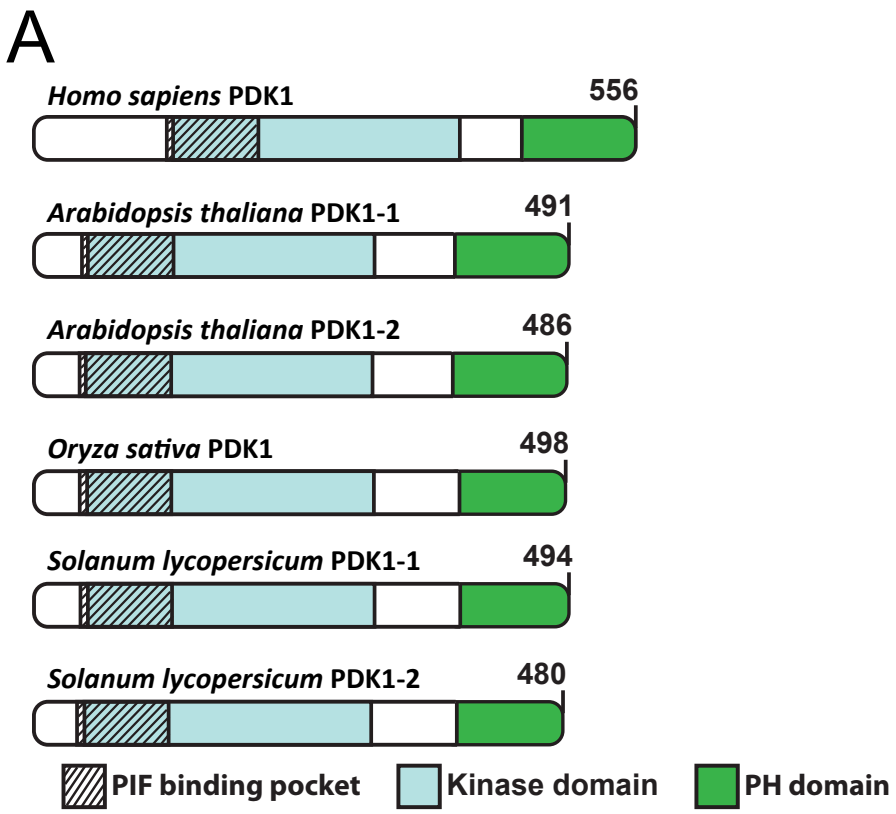

Figure 8. Protein domains and roles of PDK1 from plants. A, Diagrams of PDK1 proteins from human, *A. thaliana*, rice (*Oryza sativa*), and tomato (*Solanum lycopersicum*) with the approximate locations of functional domains within each protein. The number of amino acids is indicated at the top right corner of each protein. B, Simplified diagram of a few of the many PDK1-dependent pathways in plants, including proteins that perform some similar functions as human PDK1 substrates. Phosphatidic acid (PA) is a plasma membrane phospholipids, and can be produced in several ways. Production from the membrane phospholipids phosphatidylcholine or phosphatidylethanolamine via Phospholipase D (PLD) activity is shown (Testerink and Munnik, 2011).

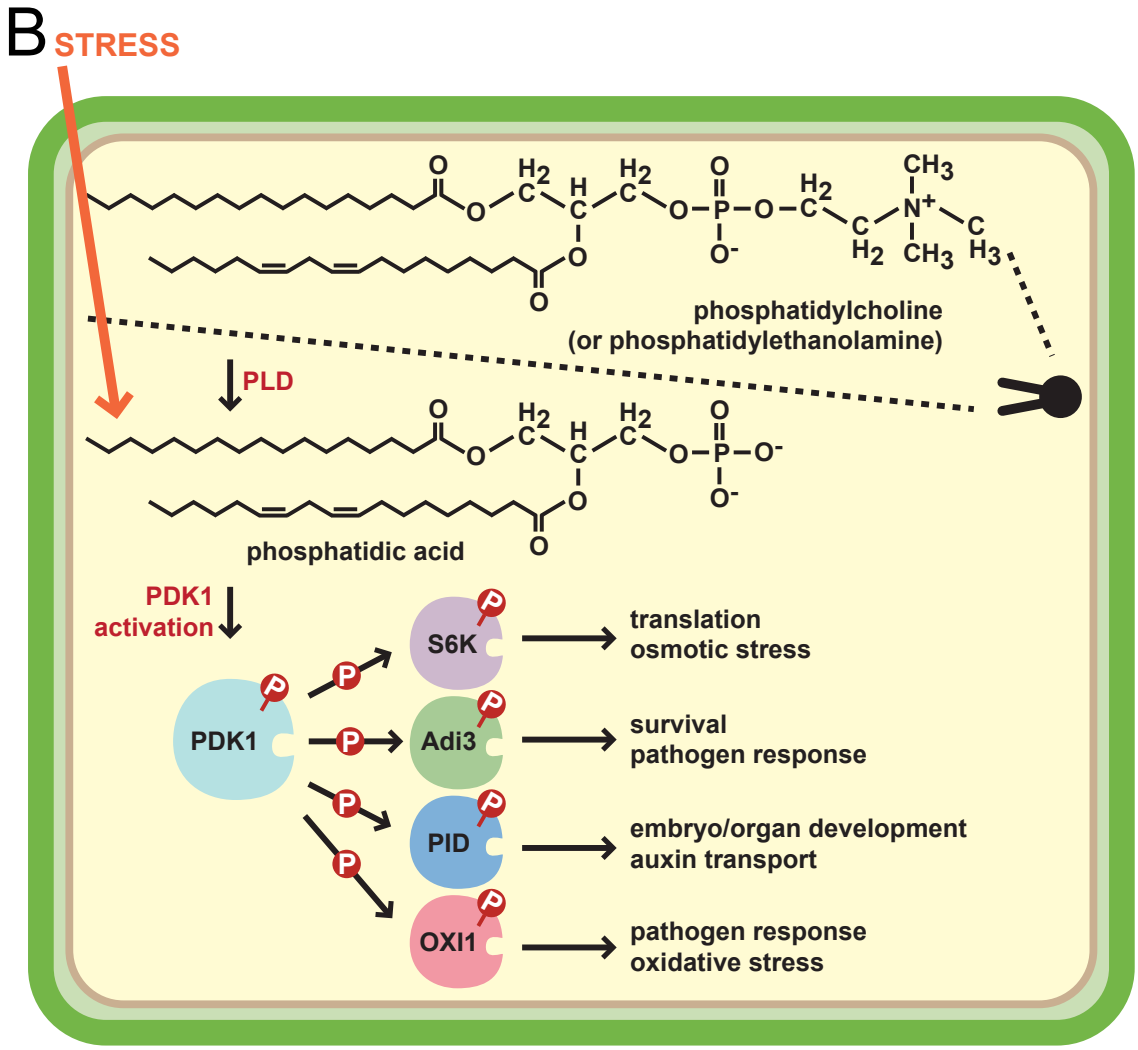

Figure 8, continued.

PA has emerged as an important plant secondary messenger that is produced in a wide variety of circumstances, such as during abiotic stress (drought, osmotic stress, cold, wounding), pathogen response, and root and pollen tube growth (Testerink and Munnik, 2011). One study found that both PtdIns(4,5)P_2 and PA increase *A. thaliana* PDK1 activity on a substrate peptide almost 2 fold, whereas PtIns3P does not (Anthony et al., 2004).

A. thaliana PDK1 is thought to be constitutively expressed (Zimmermann et al., 2004), making it a good signaling integrator that is able to control diverse processes in many tissues. The demonstrated roles for PDK1 include regulating protein translation, responses to osmotic and oxidative stress, embryo and organ development, and hormone (auxin) transport, as well as mediating the onset of programmed cell death and plant interactions with both beneficial and detrimental organisms (Fig. 8B). Several of these activities are known to occur downstream of PA signaling (Testerink and Munnik, 2011), including the regulation of beneficial (Camehl et al., 2011) and harmful (Anthony et al., 2006) biotic interactions, and also the regulation of oxidative stress response (Anthony et al., 2006), root hair growth (Anthony et al., 2004), and auxin transport (Zegzouti et al., 2006a). These results suggest that, like other PDK1s, plant PDK1s are also regulated by lipid interactions, but that the specifics differ from fungi or animals.

1.7c Conservation of PDK1 substrates in plants

Many plant AGC kinases are either postulated or confirmed substrates of PDK1. Several of these will be discussed in more detail in a later section. Some PDK1 substrates seem to be conserved between plants and mammals, but the largest plant AGC kinase

subfamily (AGCVIII, whose name comes from the initial protein kinase classification scheme of Hanks), which comprises 23 members in *A. thaliana*, is found only in plants (Bögre et al., 2003; Zegzouti et al., 2006b). Many of the *A. thaliana* AGC kinases, including the majority of the AGCVIII kinases, appear to be substrates of PDK1 (Bögre et al., 2003; Zegzouti et al., 2006b; Galvan-Ampudia and Offringa, 2007; Zhang and McCormick, 2009). Accordingly, some roles of plant PDK1 signaling are conserved with yeast and mammals, whereas many others are plant-specific functions.

1.8 PDK1 substrates in mammals

Of the 60 human AGC kinases, PDK1 is thought to phosphorylate more than 20 isoforms within the conserved activation loop site (Pearce et al., 2010). There is evidence for PDK1 phosphorylation of all 3 isoforms of Akt/PKB, all 3 isoforms of serum and glucocorticoid-induced kinase (SGK), both isoforms of ribosomal S6 kinase (S6K), all 4 isoforms of p90 ribosomal S6K (RSK), 5 of the 9 isoforms of PKC, all 3 isoforms of PKC-related kinase (PRK, also called PKN), and 2 of the 3 isoforms of PKA (Mora et al., 2004; Pearce et al., 2010; Freeley et al., 2011; Magnuson et al., 2012; Emamian, 2012; Romeo et al., 2012; Table 1). Because PDK1 is required for the activity of so many biologically important AGC kinases (Fig. 6C; see text below), it is not surprising that loss of PDK1 function is lethal in mammals.

Although PDK1 and PKB interact through PH domain-mediated colocalization at the plasma membrane (Mora et al., 2004; Pearce et al., 2010), this is not typical of PDK1-substrate interactions. Most PDK1 substrates lack lipid-binding domains and are

Table 1. List of human AGC kinases.

Human AGC kinases Protein name(s)	Number of amino acids	Evidence for PDK1 phosphorylation?
PDK1	556	Yes (intermolecular)
AKT1 / PKB alpha	480	Yes
AKT2 / PKB beta	481	Yes
AKT3 / PKB gamma	479	Yes
(p70) S6K	525	Yes
(p54) S6K2 / S6K beta	482	Some
(p90) RSK1	735	Yes
(p90) RSK2	740	Yes
(p90) RSK3	733	Yes
(p90) RSK4	745	Yes
SGK1	431	Yes
SGK2	427	Some
SGK3	496	Some
(conventional) PKC alpha	672	Yes
(conventional) PKC beta	671	Yes
(conventional) PKC gamma	697	No
(novel) PKC delta	676	Not required
(novel) PKC epsilon	737	No
(novel) PKC theta	706	Yes
(novel) PKC eta	682	No
(atypical) PKC zeta	592	Yes
(atypical) PKC iota	587	Yes
PKN1 / PRK1	942	Yes
PKN2 / PRK2	984	Yes
PKN3	889	Some
MSK1	802	No
MSK2	772	No
PKA alpha	351	Yes, but questionable relevance
PKA beta	351	Yes, but questionable relevance
PKA gamma	351	No

Table 1, continued.

Human AGC kinases Protein name(s)	Number of amino acids	Evidence for PDK1 phosphorylation?
PRKX	358	No
PRKY	277	No
PKG1	671	No
PKG2	762	No
GRK1	563	No
GRK2	689	No
GRK3	688	No
GRK4	578	No
GRK5	590	No
GRK6	576	No
GRK7	553	No
LATS1	1130	No
LATS2	1088	No
NDR1	465	No
NDR2	464	No
ROCK1	1354	No
ROCK2	1388	No
DMPK	639	No
DMPK2	1551	No
MRCK alpha	1732	No
MRCK beta	1711	No
CRIK	2027	No
MAST1	1570	No
MAST2	1798	No
MAST3	1309	No
MAST4	2444	No
MASTL	879	No
RSKL1	1066	No
RSKL2	549	No
SGK494	274	No

commonly localized in the cytoplasm (Pearce et al., 2010), and thus require a mechanism to dock with PDK1 in order to be phosphorylated. This is typically achieved by interaction between the substrate's C-terminal PIF motif and its corresponding PIF-binding pocket within the N-terminal lobe of the PDK1 kinase domain (Fig. 5). This interaction is now a well-established mechanism for stimulating PDK1 phosphorylation on most substrates (Mora et al., 2004).

1.8a Akt/PKB

Akt/PKB may be the most highly studied PDK1 substrate; it phosphorylates dozens of proteins, and its misregulation is connected with terrible human diseases including diabetes (isoform Akt2/PKBβ), cancer, schizophrenia, Huntington's disease, and stroke (isoform Akt1/PKBα) (Pearce et al., 2010; Emamian, 2012; Zhao et al., 2006). Like PDK1, both PKBα and PKBβ are constitutively expressed, whereas PKBγ is most highly expressed in the brain and testes; all 3 isoforms are phosphorylated by PDK1 (Table 1). The PKB isoforms collectively direct transcription, translation, cell survival and proliferation, insulin response, glucose metabolism, and organogenesis. In general, PKBα regulates cell growth and survival, PKBβ regulates glucose homeostasis, and PKBγ is crucial for brain development. These numerous pathways are incredibly complex, and because PKB has so many inputs and outputs it has rightly been called a central hub of cellular signaling. It would require a large amount of space to mention all the substrates of PKB, but a few particularly well-known and important ones are the ribosomal protein S6 (part of the 40S ribosomal subunit), Wee1 (participates in cell cycle regulation), Bax (induces apoptosis), glycogen synthase kinase 3 (GSK3; promotes

glycogen synthesis), the Huntingtin protein (required for normal neuronal function), and the $GABA_A$ receptor (inhibits neurotransmission) (Emamian, 2012).

1.8b SGK

SGK resembles PKB in many respects (Pearce et al., 2010; Bruhn et al., 2010. Both are activated downstream of PI3K signaling, and both proteins require coordinated phosphorylation by the same TOR-containing complex (TORC2) and then by PDK1 (Table 1). SGK signaling is typically initiated by cellular perception of growth factors or of stresses like heat and damaging radiation. Because of the similarities between SGK and PKB, it is not surprising that they affect the same processes, including cell survival, growth and proliferation, and that aberrant signaling of either kinase is correlated with cancers. The sequences of the three SGK isoforms are mostly quite similar, except SGK3 has a PtdIns3P-binding Phox homology (PX) domain. SGK isoforms phosphorylate many of the same proteins as PKB, but there is some substrate specificity for SGK. This is important because PI3K, but not PKB, is upregulated in some breast and ovarian cancers; SGK isoforms are now thought to be major contributors to tumorigenesis in at least some of these cases (Bruhn et al., 2010).

1.8c S6K

S6K is another PDK1 substrate with extensive roles within the cell, primarily related to the stress- and nutrient-dependent regulation of cell growth and size (Magnuson et al., 2012). Alterations to S6K signaling pathways are associated with cancers, diabetes, obesity, and Alzheimer's disease (Pearce et al, 2010; Magnuson et al., 2012). Most research focuses on the ubiquitously expressed S6K1 isoform, as loss of

S6K1 function results in more severe phenotypes. However, there is evidence for partial, but not complete, functional and regulatory redundancy between S6K1 and S6K2 (Martin et al., 2001; Fenton and Gout, 2011). Both isoforms require PDK1 phosphorylation of the activation loop site for full activity (Table 1), and they phosphorylate a wide array of proteins that regulate transcription, translation, cell size, mRNA processing, and protein folding. S6K substrates include a transcription factor (cAMP response element modulator; CREM), a translation initiation factor (eIF4B), a component of the mRNA cap-binding complex (CBP80/NCBP1), and a subunit of the CCT chaperonin complex (CCTβ). Similar to SGK, there is some overlap between S6K and PKB substrates, including the ribosomal protein S6, the pro-apoptotic protein BAD, and GSK3 (Magnuson et al., 2012).

1.8d RSK

RSK was initially identified by its ability to phosphorylate S6 in vitro, hence its name. Though subsequent work showed that S6K, rather than RSK, probably contributes the most to S6 phosphorylation in vivo, other important functions for RSK were soon discovered (Anjum and Blenis, 2008; Romeo et al., 2012). The 4 isoforms of RSK are very similar to each other, and consequently protein activity is regulated in the same way in all 4 isoforms. Interestingly, RSK has two catalytic domains, of which only the N-terminal catalytic domain belongs to the AGC group; this results in a relatively complex regulation. PDK1 phosphorylates the activation loop of the AGC-type kinase domain, but several other phosphorylation events must occur before and after PDK1 phosphorylation for RSK to become fully active. Once RSK isoforms have been

activated, they are capable of phosphorylating a number of proteins in both the cytoplasm and the nucleus that promote translation, cell survival, growth, and proliferation (Anjum and Blenis, 2008; Romeo et al., 2012).

1.8e PKC

PKC isoforms fall into 3 classes: conventional (α, β, and γ isoforms), novel (δ, ε, θ, and η isoforms), and atypical (ζ and ι isoforms) (Newton, 2003; Freeley et al., 2011). In addition to these, the human genome contains 3 isoforms of the PKC-related kinase (PRK, also called PKN) (Pearce et al., 2010), which closely resembles the budding yeast Pkc1. Most of the PKC and PKN isoforms are phosphorylated by PDK1 (Table 1). PKC and its relatives have various membrane-targeting domains, with which they transduce signals from insulin, growth factors, and phospholipid and Ca^{2+} secondary messengers (Roffey et al., 2009; Pearce et al., 2010). PKC isoforms are known to phosphorylate a host of substrates, including well-known proteins like the pro-apoptotic protein Bcl-2, the cAMP response element binding protein (CREB), the $GABA_A$ and glutamate receptors, mitogen activated protein kinases (MAPKs), the neutrophil NADPH oxidase, phospholipase D, and DNA topoisomerase (see www.pkclab.org). Together the PKC isoforms are required for diverse processes like insulin response, B- and T-cell mediated immune responses, central nervous system function, regulation of apoptosis, and wound healing. PKC misregulation has been noted in several cancers, heart disease, and stroke (Roffey et al., 2009; Pearce et al., 2010).

1.8f PKA

Unlike most other PDK1 substrates, the catalytic subunit of PKA is constitutively active due to constitutive phosphorylation of its activation loop. Instead, PKA activation occurs via cyclic AMP, which is produced in response to G protein-coupled receptor signaling and instigates catalytic subunit dissociation from its inhibitory binding partner, the regulatory subunit (Pearce et al., 2010). At least 2 isoforms of PKA also can be phosphorylated by PDK1 in the catalytic subunit's activation loop (Moore et al., 2002; Nirula et al., 2006; Pearce et al., 2010; Table 1), but the biological relevance of PDK1 activity on PKA is still unclear. The most compelling evidence against PDK1 being required for PKA phosphorylation comes from embryonic stem cells. When PKA was purified from stem cells completely lacking PDK1, its activation loop site was still fully phosphorylated (Williams et al., 2000). This strongly suggests that in vivo the activation loop must be targeted by either a different upstream kinase or by PKA itself. Nevertheless, other studies have made arguments that the PKA activation loop could still be phosphorylated by another kinase (Cauthron et al., 1998), potentially PDK1 (Moore et al., 2002; Nirula et al., 2006). Thus, this issue remains to be conclusively resolved.

1.9 PDK1 substrates in budding yeast

The *S. cerevisiae* genome contains 17 protein kinases belonging to the AGC group (Manning et al., 2002a; Manning et al., 2002b). Five of these have been verified as substrates of the *S. cerevisiae* PDK1 homologues Pkh1 and Pkh2: Ypk1, Ypk2 (also called Ykr2), Sch9, Pkc1, and Tpk1 (Fig. 6B). The closest mammalian homologue of Sch9 appears to be S6K, as the PIF motif of both proteins is phosphorylated by the

TORC1 complex, and both proteins are responsible for promoting translation through phosphorylation of S6 (Urban et al., 2007; Jacinto and Lorberg, 2008). Ypk1/2 are phosphorylated by the TORC2 complex, and thus appear to be homologous to PKB and SGK (Casamayor et al., 1999; Kamada et al., 2005; Jacinto and Lorberg, 2008; Niles et al., 2012). Pkc1 is the only budding yeast homologue of the 12 mammalian PKC and PKN genes (Mellor and Parker, 1998; Jacinto and Lorberg, 2008), and Tpk1 is one of three homologues of the mammalian PKA gene family (Jacinto and Lorberg, 2008). Though the genetic tractability of *S. cerevisiae* has enabled a good understanding of the biological processes each substrate of Pkh1/2 regulates, relatively little is known about the molecular mechanisms that are responsible for each outcome.

1.9a Ypk1/2

In the same report that first described Pkh1/2, Ypk1/2 were identified as SGK and (to a lesser extent) PKB homologues based on the ability of the human genes to rescue the lethal phenotype of Ypk1/2 deletion (Casamayor et al., 1999). Like SGK and PKB, both proteins are regulated through dual phosphorylation events by TORC2 and PDK1, but the substrates of Ypk1/2 still are not well understood. Two recent reports describe Ypk1 phosphorylation of Fpk1, a protein kinase that activates plasma membrane flippase enzymes (Roelants et al., 2010), and Orm1/2, which act as inhibitors of sphingolipid biosynthesis (Roelants et al., 2011). Both Ypk1 phosphorylation events are inhibitory; thus, phosphorylation of Fpk1 downregulates membrane dynamics, and phosphorylation of Orm1/2 promotes sphingolipid biosynthesis. Both reports help define the mechanisms by which Ypk1 may regulate growth and membrane dynamics (Fig. 6B), but many

questions about the full scope of Ypk1 activity still remain. As just one example, it is well known that Ypk1/2 are activated by Pkh1/2 downstream of sphingolipids produced upon heat stress (Dickson, 2008; Jacinto and Lorberg, 2008; Fig. 6B), but the downstream components of Ypk1/2 heat stress signaling are not yet established.

1.9b Sch9

Sch9 is understood to exert control over heat stress response, cell size, and aging (Dickson, 2008; Jacinto and Lorberg, 2008; Fig. 6B). Though Sch9 is now thought to be most similar to S6K (Jacinto and Lorberg, 2008), this view has only emerged in the past few years (Sobko, 2006) from the discovery that TORC1 rather than TORC2 phosphorylates Sch9 (Urban et al., 2007). Loss of Sch9 function slows cellular aging and increases stress tolerance through mechanisms that are in the process of being elucidated. Recent discoveries have shown that cells lacking Sch9 are smaller and live longer than wild-type cells, in part because ribosome biogenesis and protein synthesis are reduced. Substrates of Sch9 that participate in ribosome production include the RNA polymerase III inhibitor Maf1 (Huber et al., 2009) and the transcription factors Stb3, Dot6, and Tod6 (Huber et al., 2011). In addition, Sch9 phosphorylates the protein kinase Rim15, thereby inhibiting its ability to promote antioxidant and stress responses (Wanke et al., 2008). Other substrates of Sch9 will surely be identified in future research.

1.9c Pkc1

Pkc1 has been most studied in regard to two functions required for *S. cerevisiae* budding: stabilizing the cell wall and regulating actin dynamics during polar growth (Mellor and Parker, 1998; Levin, 2005; Jacinto and Lorberg, 2008; Fig. 6B). Loss of

Pkc1 function leads to cell lysis that can be prevented by the addition of osmotic stabilizers like sorbitol, indicating that Pkc1 is necessary for proper cell wall production. Cell wall integrity is thought to be perceived by several cell surface receptors, including Wsc1/2/3 (Levin, 2005); signaling then takes place through the Rho1 GTPase which facilitates Pkc1 activation by phosphatidylserine (Kamada et al., 1996). The most prominent Pkc1 substrate in the cell wall integrity pathway is the MAPKKK Bck1. Pkc1 promotes cell wall integrity through an activating phosphorylation of Bck1, which then activates a MAP kinase pathway that ultimately leads to cell wall biosynthesis (Levin, 2005). Interestingly, both Rho1 and Pkc1 also regulate polar cell growth by altering actin polymerization. The direct Pkc1 substrates that participate in this process have not been identified, but both Pfy1 (a profilin, which stimulates actin polymerization) and the cell cycle regulatory proteins Zds1/2 act at some point downstream of Pkc1 (Pujol et al., 2009; Anastasia et al., 2012).

1.9d Tpk1

Finally, Pkh1/2 phosphorylation of the activation loop site is thought to be at least partially responsible for proper function of the PKA homologue Tpk1 (Voordeckers et al., 2011). Tpk1/2/3 are required for proper growth in glucose-containing medium, and they also help coordinate stress responses and ribosome biogenesis (Jacinto and Lorberg, 2008). These functions are probably accomplished through the phosphorylation of many substrates that largely do not overlap with each other (Ptacek et al., 2005). Direct targets of Tpk1 include the protein kinases Yak1 (Malcher et al., 2011) and Rim15 (Reinders et al., 1998), and the cell cycle promoting protein Cdc20 (Searle et al., 2004). As with the

other AGC kinases mentioned here, it is highly likely that Tpk1/2/3 exert their effects by phosphorylating additional substrates that simply await identification.

1.10 PDK1 substrates in plants

Because the PDK1 substrates discussed in the previous two sections are critical for proper cell growth, one might expect them to be highly conserved in all eukaryotes. Interestingly, though plants have proportionally many more kinases than human or yeast, they seem to have dispensed with several of the AGC kinases that have been most highly studied in fungi and animals. In particular, no plant homologues of the growth factor responsive kinases PKB and PKC (Bögre et al., 2003) or the cyclic nucleotide-dependent kinases PKA and PKG (Bridges et al., 2005; Martinez-Atienza J et al., 2007) have been identified. If such kinases do exist, the sequences must be quite unlike their mammalian counterparts. This raises the obvious question: what are PDK1 and its AGC kinase substrates doing in plants?

Like the mammalian AGC kinases, some plant AGC kinases have been extensively investigated while others seem to have been completely ignored. The only plant whose complete AGC kinase complement has been evaluated is *A. thaliana*, which has at least 39 (Bögre et al., 2003) and possibly as many as 60 (Martin et al., 2009; Table 2; see http://www.compbio.dundee.ac.uk/kinomer/bin/kinomes.pl) AGC kinase genes. Of the 39 *A. thaliana* AGC kinases that were originally analyzed (Bögre et al., 2003), 16 belong to the AGC VI, AGC VII, AGC Other, and PDK1 subfamilies, which are also found in non-plant species; the other 23 fall into the plant-specific AGC VIII subfamily (Table 2). Because an exhaustive list of AGC kinases does not exist for any other plant,

Table 2. List of *A. thaliana* AGC kinases. This list was compiled from Bogre et al., 2003 and Martin et al., 2009. Alternate transcripts are denoted by .1, .2, and .3 after the gene number and by a, b, and c after their aliases; they are also highlighted for emphasis. Genes listed as not published in Bogre et al., 2003 were identified in the work of Martin et al., 2009, but not characterized further in that publication. Some of these gene numbers have been changed to reflect the current gene numbers at The Arabidopsis Information Resource (TAIR). Each gene's classification into an AGC subfamily or another kinase family is based on previously published work (Bogre et al., 2003) or on the classification scheme listed at WikiKinome (http://www.kinase.com/wiki/index.php/Kinase_classification). A question mark next to the putative PIF motif indicates that this PIF is based on my speculation rather than the published work of others (Bogre et al., 2003; Zegzouti et al., 2006b).

Gene number	# amino acids	Published in Bogre et al., 2003?	Alias(es)	Subfamily	Putative PIF motif
AT3G12690.1	577	Yes	AGC1-5a	AGC VIIIa	FEYF
AT3G12690.2	577	Yes	AGC1-5b	AGC VIIIa	FEYF
AT3G12690.3	577	Yes	AGC1-5c	AGC VIIIa	FEYF
AT1G16440.1	431	Yes	AGC1-6, RHS3	AGC VIIIa	FEYF
AT1G79250.1	555	Yes	AGC1-7	AGC VIIIa	FEYF
AT5G40030.1	499	Yes	AGC1-4	AGC VIIIa	FEFF
AT2G44830.1	765	Yes	AGC1-3	AGC VIIIa	FEFF
AT5G47750.1	586	Yes	AtPK5, D6PKL2	AGC VIIIa	FDFF
AT3G27580.1	578	Yes	AtPK7a, D6PKL3	AGC VIIIa	IDFF
AT3G27580.2	578	Yes	AtPK7b, D6PKL3	AGC VIIIa	IDFF
AT4G26610.1	506	Yes	AGC1-2, D6PKL1	AGC VIIIa	FDFF
AT5G55910.1	498	Yes	AGC1-1, D6PK	AGC VIIIa	FDFF
AT5G03640.1	926	Yes	AGC1-8	AGC VIIIa	FELF
AT2G36350.1	948	Yes	AGC1-9	AGC VIIIa	FELF
AT3G52890.1	934	Yes	KIPKa	AGC VIIIa	FELF
AT3G52890.2	934	Yes	KIPKb	AGC VIIIa	FELF
AT3G44610.1	451	Yes	AGC1-12	AGC VIIIa	FELF, VDYY (?)
AT2G34650.1	438	Yes	PID	AGC VIIIa	FDYF
AT2G26700.1	525	Yes	AGC1-10, PID2	AGC VIIIa	FDYF
AT1G53700.1	476	Yes	PK3, WAG1	AGC VIIIa	?
AT3G14370.1	480	Yes	AGC1-11, WAG2	AGC VIIIa	?

Table 2, continued.

Gene number	# amino acids	Published in Bogre et al., 2003?	Alias(es)	Subfamily	Putative PIF motif
AT3G12690.1	577	Yes	AGC1-5a	AGC VIIIa	FEYF
AT3G12690.2	577	Yes	AGC1-5b	AGC VIIIa	FEYF
AT3G12690.3	577	Yes	AGC1-5c	AGC VIIIa	FEYF
AT1G16440.1	431	Yes	AGC1-6, RHS3	AGC VIIIa	FEYF
AT1G79250.1	555	Yes	AGC1-7	AGC VIIIa	FEYF
AT5G40030.1	499	Yes	AGC1-4	AGC VIIIa	FEFF
AT2G44830.1	765	Yes	AGC1-3	AGC VIIIa	FEFF
AT5G47750.1	586	Yes	AtPK5, D6PKL2	AGC VIIIa	FDFF
AT3G27580.1	578	Yes	AtPK7a, D6PKL3	AGC VIIIa	IDFF
AT3G27580.2	578	Yes	AtPK7b, D6PKL3	AGC VIIIa	IDFF
AT4G26610.1	506	Yes	AGC1-2, D6PKL1	AGC VIIIa	FDFF
AT5G55910.1	498	Yes	AGC1-1, D6PK	AGC VIIIa	FDFF
AT5G03640.1	926	Yes	AGC1-8	AGC VIIIa	FELF
AT2G36350.1	948	Yes	AGC1-9	AGC VIIIa	FELF
AT3G52890.1	934	Yes	KIPKa	AGC VIIIa	FELF
AT3G52890.2	934	Yes	KIPKb	AGC VIIIa	FELF
AT3G44610.1	451	Yes	AGC1-12	AGC VIIIa	FELF, VDYY (?)
AT2G34650.1	438	Yes	PID	AGC VIIIa	FDYF
AT2G26700.1	525	Yes	AGC1-10, PID2	AGC VIIIa	FDYF
AT1G53700.1	476	Yes	AtPK3, WAG1	AGC VIIIa	?
AT3G14370.1	480	Yes	AGC1-11, WAG2	AGC VIIIa	?

Table 2, continued.

Gene number	# amino acids	Published in Bogre et al., 2003?	Alias(es)	Subfamily	Putative PIF motif
AT3G45780.1	996	Yes	PHOT1a	AGC VIIIb	?
AT3G45780.2	996	Yes	PHOT1b	AGC VIIIb	?
AT5G58140.1	915	Yes	PHOT2a	AGC VIIIb	IDLF (?)
AT5G58140.2	915	Yes	PHOT2b	AGC VIIIb	IDLF (?)
AT5G58140.3	915	Yes	PHOT2c	AGC VIIIb	IDLF (?)
AT1G51170.1	404	Yes	AGC2-3	AGC VIIIb	FVDF
AT3G20830.1	408	Yes	AGC2-4	AGC VIIIb	FIEF
AT3G25250.1	421	Yes	AGC2-1, OXI1	AGC VIIIb	FLVF
AT4G13000.1	372	Yes	AGC2-2	AGC VIIIb	FIVF
AT3G08730.1	465	Yes	AtPK6/S6K1	AGC VI	FTNFTY
AT3G08720.1	471	Yes	AtPK19/S6K2a	AGC VI	FTNFTY
AT3G08720.2	471	Yes	AtPK19/S6K2b	AGC VI	FTNFTY
AT1G48490.1	878	Yes	IRE-3a	AGC Other	FDNFSF
AT1G48490.2	878	Yes	IRE-3b	AGC Other	FDNFSF
AT3G17850.1	1296	Yes	IRE-H1	AGC Other	FSNFSF
AT1G45160.1	1067	Yes	IRE-4	AGC Other	LINFSF
AT5G62310.1	1068	Yes	IRE	AGC Other	FSNFSF

Table 2, continued.

Gene number	# amino acids	Published in Bogre et al., 2003?	Alias(es)	Classification?	Putative PIF motif
AT2G20050.1	1094	No	None	Unknown	?
AT2G20050.2	1091	No	None	Unknown	?
AT2G45490.1	288	No	AUR3	Other group	?
AT2G25880.1	288	No	AUR2a	Other group	?
AT2G25880.2	256	No	AUR2b	Other group	?
AT4G32830.1	294	No	AUR1	Other group	?
AT5G18700.1	1366	No	RUK, EMB3013	Unknown	?
AT1G50240.2	1322	No	FU	Unknown	?
AT3G12200.1	571	No	NEK7a	Other group	?
AT3G12200.2	581	No	NEK7b	Other group	?
AT3G44200.1	941	No	NEK6	Other group	?
AT3G63280.1	555	No	NEK4a	Other group	?
AT3G63280.2	555	No	NEK4b	Other group	?
AT3G59410.1	1241	No	GCN2a	Unknown	?
AT3G59410.2	1265	No	GCN2b	Unknown	?

Table 2, continued.

Gene number	# amino acids	Published in Bogre et al., 2003?	Alias(es)	Classification?	Putative PIF motif
AT2G17520.1	841	No	IRE1-2	Other group	?
AT5G24360.1	881	No	IRE1-1a	Other group	?
AT5G24360.2	887	No	IRE1-1b	Other group	?
AT5G24360.3	867	No	IRE1-1c	Other group	?
AT3G46160.1	393	No	None	Unknown	?
AT4G32250.1	611	No	None	Unknown	LVFF (?)
AT4G32250.2	611	No	None	Unknown	LVFF (?)
AT4G32250.3	611	No	None	Unknown	LVFF (?)
AT2G40560.1	303	No	None	Unknown	?
AT2G40580.1	311	No	None	Unknown	?
AT2G42550.1	344	No	None	Unknown	?
AT5G27510.1	301	No	None	Unknown	?
AT5G27790.1	327	No	None	Unknown	?
AT2G41910.1	373	No	None	Unknown	?
AT2G05060.1	315	No	None	Unknown	?

this section focuses on *A. thaliana* genes, but also discusses a few specific AGC kinases that have been reported from other plant species. However, it is important to remember that even in *A. thaliana* a number of AGC kinases have not been studied in detail.

Almost all of the 39 original AGC kinases (Bögre et al., 2003) have a putative PIF motif at or near the C-terminus of the protein (see the first 3 pages of Table 2), which can theoretically mediate interaction with PDK1. Examination of the 21 uncharacterized AGC-like kinases (Martin et al., 2009) revealed that they all lack a conventional PIF motif (see the last 2 pages of Table 2). The lack of a PIF motif is almost certainly why Bögre et al. did not consider these kinases to be AGC kinases, as a PIF motif was one of the criteria used for their classification (Bögre et al., 2003). It will nevertheless be interesting to investigate these mostly uncharacterized proteins in the future.

1.10a Plant-specific AGC kinases: AGC VIII subfamily

The AGC VIII kinases generally have a 4 amino acid "plant-type" PIF motif (Table 2; Fig. 5B), which contains an acidic Asp or Glu and enables constitutive interaction with PDK1 (assuming both proteins are in close proximity). The other AGC kinases seem to either lack a PIF altogether or have the "mammalian-type" PIF, which consists of 6 amino acids, one of which is a phosphorylatable Ser or Thr (Table 2; Fig. 5A). Proteins with a phosphorylatable PIF motif can be subjected to an additional layer of regulation; this situation is well established in mammalian systems but remains largely uncharacterized in plants.

The broadest study of AGC kinase substrates of PDK1 was performed several years ago (Zegzouti et al., 2006b), with the aim of understanding the regulation of the

plant-specific AGC VIIIa subfamily (see the first page of Table 2) by PDK1. All AGC VIIIa kinases except WAG1 and WAG2 appear to possess a "plant-type" PIF motif (Fig. 5B) comprising the C-terminal 4 amino acids of the protein, with which the AGC kinases should be able to dock with PDK1. Accordingly, of the 16 AGC VIIIa kinases investigated (AGC1-8 was not cloned), only WAG1, WAG2, and KIPK were not activated by PDK1 phosphorylation (Zegzouti et al., 2006b; Zegzouti et al., 2006a). After demonstrating that almost all AGC VIIIa kinases are substrates of PDK1, the authors also characterized the localization of some of the AGC VIIIa kinases. Most exhibit nuclear or cytoplasmic localization, but Pinoid (PID) is localized to the plasma membrane, in accordance with its ability to bind phospholipids. Thus, *A. thaliana* PDK1 is probably similar to human PDK1 in that it can interact with substrates at multiple locations in the cell through PIF-PIF binding pocket docking.

Though the plant field still lags behind mammalian systems, relatively good progress has been made in understanding the 17 AGC VIIIa kinases (Table 2), the subfamily that is currently the most well understood group of plant AGC kinases. To my knowledge, the following members of the AGC VIIIa group have not been investigated beyond the report described above (Zegzouti et al., 2006b): AGC1-4, AGC1-8, AGC1-9, KIPK, and AGC1-12. However, additional studies have been performed on the following: 1) 4 regulators of auxin transport, PID and its homologues AGC1-10/PID2, WAG1, and WAG2; 2) 4 additional auxin transport regulators, the D6 protein kinases (D6PKs), comprising AGC1-1/D6PK, AGC1-2/D6PK-like 1, AGC1-5/D6PK-like 2, and AGC1-7/D6PK-like 3; 3) 2 regulators of pollen tube growth, AGC1-5 and AGC1-7; 4) 1

regulator of root hair growth, AGC1-6; 5) 1 suppressor of programmed cell death, Adi3 (from tomato) and its closest *A. thaliana* relative, AGC1-3. Adi3 and AGC1-3 will be discussed in more detail than other AGC kinases in the following section.

1.10b Auxin transport: PID, PID2, WAG1, WAG2

PID was named because as with other plants that exhibit defective auxin production, dynamics, or signaling, plants lacking PID cannot properly conduct organogenesis, and thus produce pin-shaped inflorescences (Christensen et al., 2000; Robert and Offringa, 2008). PID regulates polar auxin transport by phosphorylating the PIN auxin efflux carriers, thereby directing polar localization of the PIN proteins to a particular side of the plasma membrane (Friml et al., 2004; Robert and Offringa, 2008). PID phosphorylation marks the PIN proteins for endosomal recycling, which is how PIN polarity is established. This recycling process enables auxin to be preferentially transported to the root and shoot apex; localized concentrations of auxin then promote (among other processes) organogenesis, phototropism in aerial tissues, and gravitropism in roots (Dhonukshe et al., 2008; Santner and Watson, 2006; Robert and Offringa, 2008). Though PID was the first AGC kinase identified as a PIN regulator, and thus is most intensely studied, recent work has shown that PID is not the only kinase to target PIN proteins. This is not surprising, because in PID loss-of-function plants not all PIN proteins are mislocalized; only the apically localized PIN proteins are affected (Robert and Offringa, 2008). Two studies have shown that PID has three close relatives with partially redundant functions: PID2/AGC1-10, WAG1/PK3, and WAG2/AGC1-11. One report found that, unlike single or double mutants, almost all (98%) *pid/pid2/wag1/wag2*

quadruple mutant plants are unable to produce cotyledons (Cheng et al., 2008), indicating that these 4 AGC kinases share responsibility for mediating polar PIN localization. Another study showed that PID, WAG1, and WAG2 all target PIN proteins to polar endosomal recycling pathways (Dhonukshe et al., 2010), providing further evidence for the partial functional redundancy that had been previously reported (Cheng et al., 2008). It is interesting to note that both PID and PID2 have a putative PIF motif at the C-terminus, and thus can be regulated by PDK1 phosphorylation, whereas WAG1 and WAG2 do not (Table 2). One group has investigated the mechanism and significance of PID phosphorylation by PDK1 (Zegzouti et al., 2006a), but further work still remains to be done.

1.10c Auxin transport: D6PK, D6PKL1, D6PKL2, D6PKL3

Similar to PID and its three homologues, AGC1-1/D6PK and its homologues AGC1-1/D6PK, AGC1-2/D6PKL1, AGC1-5/D6PKL2, and AGC1-7/D6PK3 also are thought to regulate polar auxin transport. The D6PKs are capable of phosphorylating PIN proteins in vitro and colocalize with some PIN proteins at the rootward plasma membrane in root cells (Zourelidou et al., 2009). However, in contrast to the PID homologues, the D6PK homologues do not appear to control PIN polar localization (Dhonukshe et al., 2010). Thus, the mechanisms by which D6PKs regulate auxin transport are still unclear; perhaps other auxin transport proteins (such as the AUX and LAX auxin influx carriers) are the targets of D6PKs in vivo. All 4 D6PK homologues have a C-terminal sequence that resembles the “plant-type” PIF motif (Table 2; Fig. 5B), possibly indicating that they are targets for PDK1 phosphorylation. One report found

that PDK1 can activate all 4 proteins in vitro (Zegzouti et al., 2006b), but the significance of this has not been investigated in detail. Furthermore, a different study showed that PDK1 does not activate D6PK (Anthony et al., 2004), so this situation should be clarified in the future.

1.10d Pollen tube growth: AGC1-5, AGC1-7

AGC1-5 and AGC1-7 are primarily expressed in pollen, and they appear to play redundant roles in regulating the polarity growth of pollen tubes. In *agc1-5/agc1-7* double mutant plants (but not single mutant plants), growth along an axis is compromised, and the cells appear distorted with many bulges in the plasma membrane. In accordance with this result, actin filaments are disrupted in mutant pollen tubes, indicating that AGC1-5 and AGC1-7 enable polar growth by promoting organized placement of cytoskeletal elements (Zhang et al., 2009). Unfortunately, no substrates of AGC1-5 and AGC1-7 have been identified yet. Both kinases have a "plant-type" PIF motif (Table 2; Fig. 5B) and are activated by PDK1 in vitro (Zegzouti et al., 2006b), but the significance of AGC1-5 and AGC1-7 phosphorylation by PDK1 has been questioned. A recent review has reported that the tips of pollen tubes are misshapen when constitutively active AGC1-5 (harboring a Ser-to-Asp point mutation to mimic a phosphorylated activation loop) is overexpressed. In contrast, overexpression of PDK1 does not produce a detectable pollen tube phenotype (Zhang and McCormick, 2009). However, this could be explained by the fact that PDK1 activity can be counteracted by dephosphorylation, whereas an Asp in the activation loop is irreversible. Hopefully these possibilities will be investigated in future experiments.

1.10e Root hair growth: AGC1-6

AGC1-6/RHS3 is a close relative of AGC1-5 and AGC1-7, and interestingly these 3 kinases are found in cell types that exhibit polar growth; however, AGC1-6 expression is confined to root hairs rather than pollen tubes (Won et al., 2009; Zhang et al., 2009). Like AGC1-5, overexpression of RHS3 produces defects in cell morphology (Won et al., 2009). Not much else is known about RHS3 function, except that it has a "plant-type" PIF motif (Table 2; Fig. 5B) and is activated by PDK1 in vitro (Zegzouti et al., 2006b). No substrates have been reported so far, and the contribution of PDK1 to RHS3 function is still unknown.

1.10f Other confirmed AGC kinase substrates of PDK1

Besides kinases from the AGC VIIIa subfamily, at least 2 other plant AGC kinases have also been confirmed as targets of PDK1 phosphorylation. These include: 1) a regulator of reactive oxygen species (ROS) signaling, AGC2-1/OXI1 (from the AGC VIIIb subfamily); 2) a regulator of sugar signaling, S6K (from the AGC VI subfamily). In contrast to the AGC VIII kinases discussed for the majority of this section, S6K has the "mammalian-type" PIF motif, which contains a phosphorylatable Ser or Thr (Table 2; Fig. 5A).

1.10g Oxidative burst signaling, root hair growth: OXI1

AGC2-1/OXI1 was first described as a substrate of PDK1 that promotes the growth of root hairs (Anthony et al., 2004). In addition to regulating root growth, OXI1 is activated by both wounding and cellulase perception (Rentel et al., 2004), and mediates two distinct but related aspects of defense signaling: basal immune responses to the

oomycete pathogen *Peronospora parasitica* (Rentel et al., 2004) and growth promotion in the presence of the beneficial fungus *Piriformospora indica* (Camehl et al., 2011). The common theme in the diverse activities performed by OXI1 (root hair growth, interactions with other organisms, response to wounding) is that they all involve the production of secondary messengers: the phospholipids PA and PtdIns(4,5)P_2 (Anthony et al., 2004; Camehl et al., 2011), and ROS such as hydrogen peroxide (Rentel et al., 2004). These molecules then activate OXI1 signaling, most likely through indirect means. Activated OXI1 is thought to function by initiating MAP kinase signaling, possibly through its phosphorylation of the downstream protein kinase PTI-2 (Anthony et al., 2006). OXI1 probably has other substrates in addition to PTI-2, since at least one close relative of PTI-2, PTI-1, is phosphorylated by OXI1 in vitro (Anthony et al., 2006); however this possibility has not yet been investigated in more detail.

1.10h Nutrient and stress signaling: S6K

A. thaliana S6K is similar to the mammalian S6K in several respects. First, it responds to changes in the cellular environment, such as nutrient status (Xiong and Sheen, 2012) and cold, salinity (Mizoguchi et al., 1995), or osmotic (Mahfouz et al., 2006) stress. Second, it is regulated by both PDK1 phosphorylation of the activation loop (Mahfouz et al., 2006) and TOR phosphorylation of the PIF motif (Xiong and Sheen, 2012). Third, it phosphorylates S6 (Bögre et al., 2003), which is probably one mechanism by which S6K controls cell size and proliferation in nutrient-deprived conditions (Henriques et al., 2010). Interestingly, though several *A. thaliana* AGC kinases have the phosphorylatable “mammalian-type” PIF motif sequence (Table 2), to

my knowledge S6K is the only one for which TOR phosphorylation of the PIF site has been demonstrated (Xiong and Sheen, 2012). No research has tested whether the S6K-PDK1 interaction is dependent on a TOR-phosphorylated PIF motif, but given the other similarities between plant and animal S6Ks, it would not be surprising to find that only a phosphorylated PIF motif can dock with PDK1.

1.10i AGC kinases not confirmed as PDK1 substrates

Finally, a number of AGC kinases with a putative PIF motif (for example, AGC2-3 with a "plant" PIF, and IRE and NDR-1 with a "mammalian" PIF) are putative targets of PDK1 and/or TOR phosphorylation, but were not mentioned in this section because no published literature has reported them as PDK1 substrates (Table 2). For example, the only study of *A. thaliana* IRE found that it is expressed in both root hairs and pollen, and that loss-of-function mutant plants display significantly reduced root hair elongation (Oyama et al., 2002). The IRE orthologue in *M. truncatula*, MtIRE, is not found in root hairs but instead is predominantly expressed in root nodules (Pislariu and Dickstein, 2007). Since both *A. thaliana* and *M. truncatula* are expected to possess several IRE-like kinases, it is possible that one or more genes has retained a conserved function in both plants, while others are able to evolve new roles due to organism-specific selective pressures. In contrast to kinases like IRE, which are predicted substrates of PDK1 even though experimental evidence is lacking (Pislariu and Dickstein, 2007), the AGC-like kinases identified by Martin et al., 2009 generally seem to lack a readily identifiable PIF motif. Thus, though the roles of these kinases are also largely unknown, they will probably not be considered likely candidate substrates of PDK1 (Table 2). Of course,

AGC kinases without a PIF might still interact with PDK1 through alternative mechanisms. In the future it would be interesting to investigate similarities and differences in the functional and regulatory features of the many unstudied AGC kinases.

1.11 Adi3, a plant-specific regulator of programmed cell death

The tomato AGC VIIIa kinase Adi3 was identified in a screen for proteins that interact with both AvrPto, a virulence protein from the plant-pathogenic bacterium *Pseudomonas syringae*, and Pto, the tomato protein that recognizes AvrPto and instigates defense signaling (Bogdanove and Martin, 2000). Like the AGC kinases discussed in the previous sections, Adi3 is activated by PDK1 phosphorylation of its conserved activation loop site at Ser539 (Devarenne et al., 2006; Fig. 5B).

1.11a Roles of Adi3 in plants

Similar to several mammalian AGC kinases including PKB, SGK, and S6K, Adi3 has been demonstrated to promote cell survival (Devarenne et al., 2006; Ek-Ramos et al., 2010). However, there are several differences between Adi3 and these mammalian kinases: 1) TOR regulation of Adi3 is unlikely to occur since its PIF motif lacks a phosphorylatable amino acid (Table 2); 2) Adi3 functions outside of cell death suppression have not yet been investigated; 3) the mechanisms of Adi3 cell death suppression are probably not exactly the same as PKB and S6K, which inhibit apoptosis (Emamian, 2012; Magnuson et al., 2012), a process that does not occur in plant cells (van Doorn and Woltering, 2005; van Doorn et al., 2011). Current work in the Devarenne lab focuses on studying the mechanisms by which Adi3 is able to regulate cell survival and death in plants.

Adi3 suppresses the occurrence of plant programmed cell death (PCD) (Devarenne et al., 2006; Ek-Ramos et al., 2010). When Adi3 is downregulated by virus-induced gene silencing, plant growth is stunted and localized regions of dead cells spontaneously appear on both stems and leaves, indicating that Adi3 inhibits PCD in wild-type plants. Furthermore, overexpression of constitutively active Adi3 partially attenuates cell death induced by Pto or the PDK1 inhibitor OSU-03012, providing further evidence that Adi3 acts downstream of Pto and PDK1. Co-silencing of Adi3 and a MAPKKK abolishes the appearance of PCD lesions, suggesting that they regulate PCD through similar pathways; however, Adi3 and the MAPKKK do not directly interact (Devarenne et al., 2006). Though the type of plant cell death regulated by Adi3 has not been investigated, Adi3 interacts with the autophagy-related proteins Atg8a, Atg8f, and Atg8h. Co-silencing of Adi3 and several autophagy proteins exacerbated the tomato cell death phenotype (Devarenne, 2011), which may suggest a role for autophagy in limiting the extent of cell death caused by loss of Adi3 function.

1.11b Current model for Adi3 function

The mechanism by which Adi3 regulates PCD has not been fully elucidated; in particular, not much is known about how Pto modulates Adi3 function during plant immune responses. However, recent work has provided insight into Adi3-mediated PCD suppression (Ek-Ramos et al., 2010). Adi3 harbors a nuclear localization signal within its activation loop and a nuclear exportation signal near the N-terminus of the protein; these sequences enable Adi3 to translocate into and out of the nucleus. A point mutation in Adi3 that mimics PDK1 phosphorylation (S539D) is predominantly localized to the

nucleus, the location where Adi3 is competent to suppress PCD. However, when the nuclear localization signal of Adi3 is deleted, Adi3 localizes to punctate structures in the cell (Ek-Ramos et al., 2010), which recent work suggests belong to the endosomal trafficking network.

From these experiments, a model for Adi3 function is summarized in Figure 9. In this model, in healthy cells PDK1 phosphorylates Adi3, promoting its activation and translocation to the nucleus. Fully active Adi3 is then able to phosphorylate proteins that inhibit PCD. On the other hand, pathogen attack induces plant defense responses, which causes localized PCD by preventing Adi3 from trafficking to the nucleus (Ek-Ramos et al., 2010; Oh and Martin, 2011; Fig. 9).

1.11c Adi3 homologue and substrate

The closest sequence relative of Adi3 in *A. thaliana* is AGC1-3 (Table 2), and its function is currently being characterized (Gray and Devarenne, 2012). PDK1 indeed is able to activate AGC1-3 through phosphorylation of the activation loop, as has been shown previously (Zegzouti et al., 2006b). However, no homozygous *agc1-3* mutant plants have been characterized, so it is impossible to say whether AGC1-3 is the functional homologue of Adi3. A future goal is to genetically characterize AGC1-3.

One substrate of Adi3 has been identified: Gal83, which is a regulatory subunit of the heterotrimeric sucrose non-fermenting related kinase (SnRK) complex (Avila et al., 2012). There is evidence that Adi3 phosphorylation of Gal83 may alter activity and

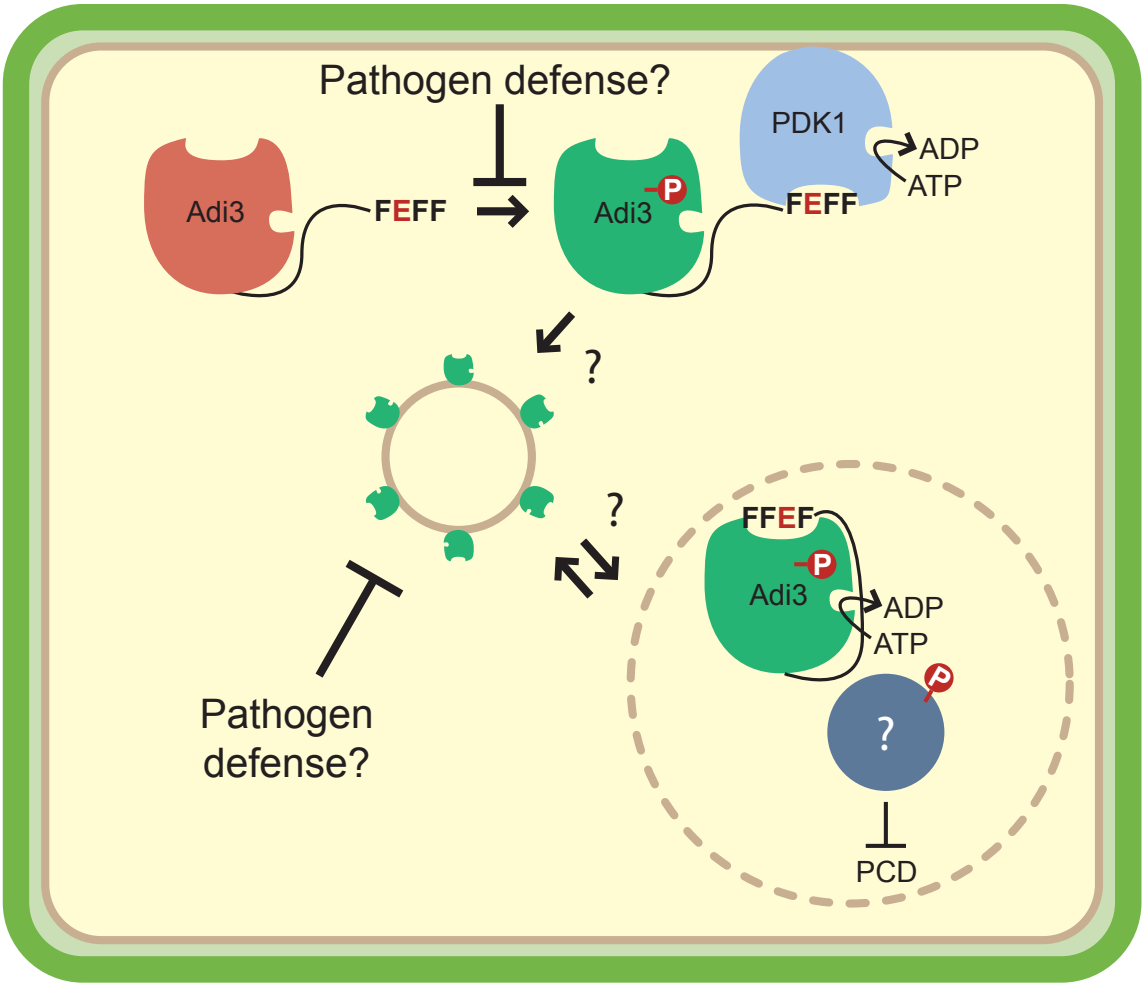

Figure 9. Current model for Adi3 function. In healthy cells, PDK1 phosphorylates Adi3, which promotes its translocation to the nucleus, possibly by interaction with a component of the endomembrane system. Nuclear-localized Adi3 is competent to suppress PCD through phosphorylation of unidentified substrate proteins. During immune responses against pathogens like P. syringae, PCD is induced, possibly by inhibiting the movement of Adi3 from the cytoplasm and/or plasma membrane to the nucleus. Question marks indicate areas of uncertainty that are being investigated by the Devarenne lab.

localization of the SnRK complex, but the link between Adi3 phosphorylation of Gal83 and PCD suppression is not fully understood.

1.12 Methods for protein kinase substrate discovery

Yeast two-hybrid screening is a common technique for discovering novel protein-protein interactions; unfortunately it is not ideal for identifying kinase substrates. Though some kinase-substrate pairs reliably interact in a yeast two-hybrid assay (e.g. Devarenne et al., 2006; Avila et al., 2012), not all interactions are persistent enough to trigger activation of the reporter gene. For example, a yeast two-hybrid screen has identified a number of Adi3 interacting proteins; only 1 of them, Gal83, so far appears to be a substrate of Adi3 (Avila et al., 2012).

One interesting technology that has proved useful for assessing the sequence requirements and specificity of a particular kinase is a chip to which an array of many potential kinase substrates is bound, either in the form of immobilized peptides (Houseman et al., 2002) or proteins (Zhu et al., 2000; Ptacek et al., 2005). The kinase is incubated with the chip, and phosphorylation of each substrate on the chip is assessed by some means, for example the incorporation of ^{32}P (Houseman et al., 2002; Ptacek et al., 2005) or ^{33}P (Zhu et al., 2000) in the substrate peptide or protein. Analysis of many phosphorylation events is an important step toward 1) learning the complete body of proteins phosphorylated by a kinase and 2) understanding why a kinase is capable of phosphorylating the proteins it does.

Sometimes kinase substrates may be identified by comparing the phosphoproteome of wild-type cells with that of cells lacking, via either knockout/knockdown or chemical

inhibition, the kinase of interest (Huber et al., 2009; Howden et al., 2011). These studies often incorporate quantitative phosphoproteomics techniques (Derouiche et al., 2011; Kosako and Nagano, 2011). Though phosphoproteomics is a powerful technique with which to investigate signaling pathways, it has a few drawbacks. First, because only the presence or absence of phosphorylation events is being measured, it is generally not possible to distinguish between direct substrates and targets that are farther downstream of the kinase of interest. Second, some null alleles of kinases are lethal, making it impossible to propagate stable knockout lines, though downregulation of the kinase might be feasible. Third, though some drugs (e.g. rapamycin) are highly specific for a single kinase (Jacinto and Lorberg, 2008; Hughes and Kennedy, 2012), many kinase inhibitors are nonselective. One difficulty in inhibitor design is that most kinase catalytic domains are highly conserved (Dittmeyer and Schnittger, 2011; Fig. 4).

To address these issues, an innovative technique to specifically identify direct kinase substrates was introduced 15 years ago (Shah et al., 1997). A point mutation is generated within the active site of the kinase of interest; the mutation enables the kinase to use an ATP analogue that has a bulky substitution at the N6 position of adenosine. The most important advantage of this technique is that bulky ATP analogues are not effective substrates for endogenous kinases. Thus, phosphorylation of cell lysate with a radiolabeled ATP analogue can result in very low background incorporation of radiolabel. These experiments selectively detect the direct substrates of an analogue-sensitive kinase, rather than a mix of direct substrates and proteins that are merely phosphorylated as a result of that pathway. The analogue-sensitive technology has been

successfully used to identify hundreds of kinase substrates in a single large scale experiment (Ubersax et al., 2003). Several modifications to the analogue-sensitive kinase method have been used successfully, including inhibitors that selectively target analogue-sensitive kinases (Bohmer and Romeis, 2007) or the incorporation of a second mutation that prevents lethality when some analogue-sensitive kinases replace the endogenous kinase in the genome (Zhang et al., 2005).

1.13 Some model plant systems: *A. thaliana*, tomato, moss

A. thaliana is a dicot belonging to the family Brassicaceae in the eurosids II clade (Fig. 10). Other members of Brassicaceae include broccoli, turnip, cabbage, radish, and horseradish. *A. thaliana* is by far the preeminent model plant; it boasts a rapid generation time, hundreds of fully sequenced accessions and hundreds more currently being sequenced (see http://www.1001genomes.org/), and vast collections of genetic tools including thousands of different transgenic lines. One of the few drawbacks of using *A. thaliana* is that its biology is not always representative of economically important crop plants, particularly monocot cereal crops like rice, wheat, and corn (Fig. 10).

Tomato is an economically and nutritionally important crop and an established model for studying plant-pathogen interactions. Unlike *A. thaliana*, which is a rosid, tomato and its relatives tobacco, potato, pepper, eggplant, and nightshade (in the family Solanaceae) are in the asterids clade (Hedges et al., 2002; Fig. 10). Surprisingly, though a number of economically important plants are asterids, only the potato genome has been published (Potato Genome Sequencing Consortium, 2011). The genomes of several other asterids, including the model plant *Mimulus guttatus* (see www.phytozome.net)

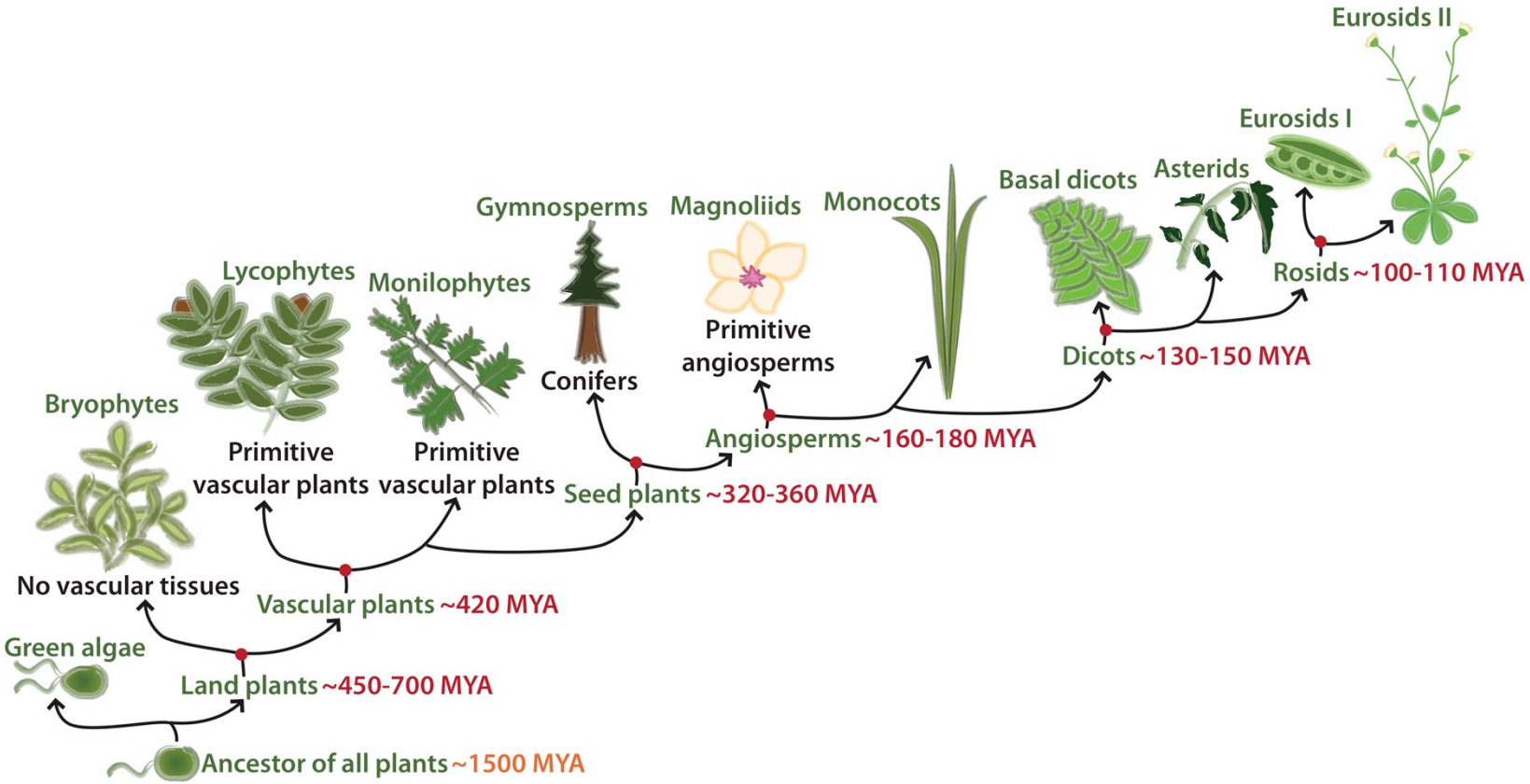

Figure 10. Simplified evolutionary history of plants (Archaeplastida). Archaeplastida is believed to be a monophyletic groups consisting of green algae and land plants. Approximate times for a few of the major events in plant evolution are shown in red. The Archaeplastida ancestor probably appeared approximately 1.5 billion years ago, long before plants colonized the land. The earliest land plants lacked vascular tissues, but later plants evolved a vascular system and reproduction by seeds rather than spores. The angiosperms (flowering plants) evolved relatively recently, but today they are the most diverse group of plants, with hundreds of thousands of extant species. The majority of economically important and model plant species are angiosperms.

and several species of tomato, are either completely sequenced or in the process of being sequenced (see http://solgenomics.net/), but they have not yet been published. Though it is feasible to generate stably transformed lines of tomato, the process is much more time consuming than in *A. thaliana*, partially due to longer generation times (months rather than weeks) and partially due to the fact that *A. thaliana* can be transformed using the rapid floral dip procedure. Related to this issue, genetic resources like large T-DNA collections are lacking for tomato. Therefore, for many applications *A. thaliana* remains a more attractive system in which to study diverse aspects of plant biology.

In recent years the moss *Physcomitrella patens* has emerged as an exciting new model plant, possessing several advantages over other models that make it an attractive experimental system. First, *P. patens* has a relatively small (~480 Mb, larger than rice but smaller than tomato), sequenced genome. Second, *P. patens* spends the majority of its life cycle in the haploid gametophyte stage, so generation of null alleles is simpler than in diploid plants (Rensing et al., 2008). Third, homologous recombination in *P. patens* is thousands of times more efficient than in flowering plants like *A. thaliana*, making targeted gene disruption and replacement possible for the first time in a plant (Schaefer, 2002). Finally, *P. patens* is a useful model in which to study plant evolution. Bryophytes, consisting of mosses, liverworts, and hornworts, are the closest extant relatives of early land plants (Fig. 10), which first appear in the fossil record about 440-460 million years ago (Wellman et al., 2003; Wellman, 2010). Molecular clock studies estimate that the bryophyte- vascular plant divergence occurred as early as 700 million years ago, suggesting that bryophytes may be even older than fossil records indicate

(Heckman et al., 2001; Hedges, 2002). Most plant models are angiosperms, a much more recent lineage (Hedges et al., 2002; Soltis et al., 2008; Fig. 10). Some characteristics of *P. patens* that might be considered advantages, including its morphological simplicity and ability to be propagated vegetatively, might be considered disadvantages in some situations. For example, its simple body plan, which lacks vascular tissues, true roots, and flowers/seeds, could make it difficult to translate knowledge about a gene's function in *P. patens* to a more complex flowering plant. However, it is still worthwhile to study the biology of *P. patens* for several reasons, one of which is its ability to uniquely illuminate the evolutionary changes that have occurred since the ancestors of modern plants first emerged from aquatic habitats to colonize the land.

1.14 Dissertation overview

I begin by describing in Chapter II all the methods used to conduct the studies discussed in Chapters III-VI. Each method in Chapter II has the chapter with which it is associated in parentheses. Next, Chapter III reports the characterization of PpPDK1, a homologue of PDK1 from *P. patens*. Unlike *A. thaliana* or tomato but like rice, PDK1 appears to be a single-copy gene in *P. patens*, and in contrast to all these vascular plants, PpPDK1 lacks a lipid-binding domain. Nevertheless, PpPDK1 can phosphorylate AGC kinases from tomato and *P. patens* in their activation loop. Mutagenesis of PpPDK1 PIF-binding pocket residues reduces kinase activity and substrate interaction. When PpPDK1 is knocked out by homologous recombination-mediated gene replacement, the moss is viable but severely compromised in its growth. This study provides a foundation for dissecting the conserved and lineage-specific functions of plant PDK1s.

In Chapter IV I compare putative PDK1 sequences from 100 distantly related photosynthetic and non-photosynthetic species and discuss possibilities for PDK1 function and evolution. Interestingly, though almost all experimentally confirmed PDK1s quite closely resemble the human PDK1, I found a relatively high degree of diversity in PDK1 size (ranging from approximately 300 to 2000 amino acids) and protein domain architecture. Particularly striking was the lack of a lipid-binding domain in a number of PDK1s, including species of fungi, algae, and diatoms. This is surprising because almost all PDK1s reported so far are regulated by lipid interactions. My results suggest that PDK1 evolutionary history is probably quite complex, and PDK1 may have a number of interesting functional differences that have not yet been investigated.

Chapter V presents results that suggest the existence of a second PDK1 phosphorylation site on Adi3, at Ser212 outside the kinase domain. This site was first identified in the *A. thaliana* sequence homologue of Adi3, AGC1-3. I confirmed that PDK1 also phosphorylates Adi3 at this site in vitro, and that while phosphorylation does not increase Adi3's autocatalytic activity, it may increase Adi3's activity on a substrate. Finally, I show that it may be possible to resolve individual differentially phosphorylated forms of Adi3 using protein gels with reduced ratios of bisacrylamide crosslinker. These results do not conclusively demonstrate PDK1 phosphorylation on Ser212, but they provide tools with which to further investigate this potential phosphorylation event.

In Chapter VI I discuss my attempts to produce a form of Adi3 that would be suitable for substrate searches using bulky ATP analogues. This method has been used successfully to identify direct substrates of a number of mammalian and yeast protein

kinases. My work identifies a “gatekeeper” amino acid that enables Adi3 to selectively use a bulky ATP analogue, and also shows that a known substrate of Adi3 is highly phosphorylated by analogue-sensitive Adi3 in vitro. Thus, in principle analogue-sensitive Adi3 should be perfectly suited to a substrate search using plant tissue extracts. Unfortunately, many attempts to transfer this system to tomato leaf extracts were unsuccessful. I speculate that plants might possess an ATPase or other enzyme that is capable of removing the gamma phosphate from ATP, resulting in high background phosphorylation and/or low analogue-sensitive kinase phosphorylation.

CHAPTER II

METHODS*

2.1 Cloning and site directed mutagenesis (Chapter III)

PpPDK1 (Pp1s217_11V6.2 at Phytozome, GenBank accession JN049607), *Pp188412* (Pp1s118_230V6 at Phytozome, GenBank accession JN049610), and *Pp174181* (Pp1s4_386V6.1 at Phytozome, GenBank accession JN049609) coding sequences were identified by searching Phytozome using the *AtPDK1* sequence (GenBank accession AF132742.1) (Deak et al., 1999). *Pp2484* (Pp1s224_73V6.1 at Phytozome, GenBank accession JN049608) was identified by searching Phytozome using the *Adi3* sequence (GenBank accession AY849914) (Devarenne et al., 2006). Total RNA was extracted from *P. patens* and *A. thaliana* tissue using TRIzol Reagent (Invitrogen) and reverse transcription was performed using SuperScriptIII reverse transcriptase (Invitrogen). Full length cDNAs for the following genes were amplified from 1st strand cDNA using forward primers beginning at the ATG start codon and reverse primers beginning at the

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*Portions of the following articles have been reprinted with permission: (1) **Dittrich ACN, Devarenne TP** (2012) An ATP analog-sensitive version of the tomato cell death suppressor protein kinase Adi3 for use in substrate identification. Biochimica et Biophysica Acta Proteins and Proteomics **1824:** 269-273. Copyright 2012 © Elsevier. (2) **Dittrich ACN, Devarenne TP** (2012) Characterization of a PDK1 homologue from the moss Physcomitrella patens. Plant Physiology **158:** 1018-1033. Copyright 2012 © American Society of Plant Biologists. (3) **Dittrich ACN, Devarenne TP** (2012) Perspectives in PDK1 evolution: insights from photosynthetic and non-photosynthetic organisms. Plant Signaling and Behavior, in press. Copyright 2012 © Landes Bioscience.

stop codon or last amino acid; *PpPDK1*, forward primer 5'-ATGGCCATGGATGGGACC-3', reverse primer 5'-TACGTCGTATACAAATGCATCCAAACC-3'; *Pp188412*, forward primer 5'-ATGGCGGCAACAAATCGCATTAAC-3', reverse primer 5'-CTATGATGAGCCCAGCCACG-3'; *Pp174181*, forward primer 5'-ATGACACTTGCTACCACTTTCAGG-3', reverse primer 5'-TTATGAGGATTCCCCACTCTTGAG-3'; *AtPDK1*, forward primer 5'-ATGTTGGCAATGGAG-3', reverse primer 5'-GCGGTTGTGAAGAGTC-3'. The annotated full-length 2,328 nt *Pp2484* cDNA could not be amplified from first strand cDNA. Instead, a 1,560 nt cDNA for *Pp2484* was amplified from first strand cDNA using forward primer 5'-ATGAGTGGAAAGTTGAGCATGAG-3', reverse primer 5'-TCAAAAAAAGTCAAAATCCACGTATGAC-3'. The amplified 1,560 nt *Pp2484* cDNA lacks the first 768 nt of exon 1 but the encoded protein contains all conserved AGC kinase elements. Cloning of *SlPDK1* and *Adi3* was previously reported (Devarenne et al., 2006). *PpPDK1*, *AtPDK1*, and *SlPDK1* lacking the endogenous stop codon were cloned into pET22-b(+) (Novagen) to incorporate a C-terminal 6His tag. *PpPDK1-6His*, *AtPDK1-6His*, and *SlPDK1-6His* were amplified from pET22-b(+) and cloned into pMAL-c2x (New England Biolabs) for N-terminal MBP translational fusions. *Pp188412*, *Pp147181*, and *Pp2484* without a 6His tag were also cloned into pMAL-c2x. Site-directed mutagenesis was performed on genes cloned into pMAL-c2x using Pfu Turbo (Stratagene) according to the manufacturer's instructions. Finally, *MBP-PpPDK1-6His*, *MBP- AtPDK1-6His*, *MBP-SlPDK1-6His*, *MBP-Pp188412*, *MBP-Pp174181*, and

MBP-PpPDK1-6His point mutants were cloned into the plasmid p416GPD (Mumberg et al., 1995) for yeast complementation studies. p416GPD contains a URA3 marker and a constitutive GPD promoter to drive expression of the gene of interest.

2.2 Yeast strains and complementation (Chapter III)

Two previously described strains of *S. cerevisiae* were used in complementation experiments: strain AC306 (Casamayor et al., 1999) was used for tetrad analysis of *PKH1/2* complementation and strain INA106-3B (Inagaki et al., 1999) was used for temperature sensitivity *PKH1/2* complementation. AC306 is a diploid strain that is heterozygous for *PKH1* and *PKH2* deletion (genotype: MATa/MATα *PKH1/pkh1Δ::HIS3 PKH2/pkh2Δ::TRP1 ade2-1 can1-100 his3-11,15 leu2-13,112 trp1-1 ura3-1*). INA106-3B is a haploid strain that lacks *PKH2* and contains a point mutation in *PKH1* (genotype: MATa $pkh1^{D398G}$ *pkh2Δ::LEU2 ade1 his3-2 trp1 ura3*). Both strains were transformed with the *PDK1* constructs in p416GPD. After transformation, AC306 yeast transformed with the desired plasmid was grown at 30°C on plates lacking uracil. Sporulation was induced by incubating transformed yeast in 1% potassium acetate at 25°C for 7 days. After sporulation, tetrad dissections were performed on at least 30 tetrads of each sample using standard techniques. After dissection, spore genotype and viability were analyzed by replica plating on minimal medium and on medium lacking histidine, tryptophan, or uracil. After spore analysis, representative cultures of each haploid spore were grown at 30°C in liquid YPD medium and spotted on both YPD plates and plates containing 5-FOA (Research Products International), which selects for loss of the URA3-marked p416GPD plasmid.

For temperature sensitive *PKH1/2* complementation, liquid cultures of INA106-3B yeast transformed with the desired plasmid were grown at 25°C in liquid medium lacking uracil and spotted on two identical plates lacking uracil. One plate was incubated at 25°C for 4 days and the other plate was incubated at 35°C for 2 days, then plates were viewed and photographs taken. To analyze the expression of all genes cloned into p416GPD, total protein was extracted as previously described (Yaffe and Schatz, 1984) from yeast grown at 30°C in liquid YPD medium (AC306 yeast) or 25°C in liquid medium lacking uracil (INA106-3B yeast). MBP fusion proteins were then detected by α-MBP (1:10,000; New England Biolabs) western blot.

2.3 In vitro kinase assays (Chapter III)

*Pp*PDK1-6His, *Pp*2411, *At*PDK1-6His, *Sl*PDK1-6His, *Pp*2411, and Adi3 were expressed as MBP fusion proteins using pMAL-c2 in *E. coli* BL21(DE3) and purified with amylose resin (New England Biolabs) according to the manufacturer's instructions. In vitro kinase assays were performed by combining the purified proteins in a 30 µl final volume of kinase buffer containing 10 mM Tris pH 7.5, 10 mM $MgCl_2$ or 10 mM $MnCl_2$, and 1 mM DTT. The reactions were started by the addition of 1 µCi γ-[^{32}P]ATP and nonradiolabeled ATP to a final concentration of 20 µM followed by incubation at room temperature for 15 min. Reactions were stopped by the addition of 4x SDS-PAGE sample buffer. Protein phosphorylation was visualized by phosphorimager (Bio-Rad Molecular Imager) after separation by SDS-PAGE and signals were quantified using Quantity One software (Bio-Rad). All kinase assays were performed a minimum of three times, with representative images shown in figures.

2.4 In vitro pulldown assays (Chapter III)

MBP, MBP-*Pp*2411, and MBP-*Pp*PDK1-6His were purified with amylose resin as described above. Five µg of purified MBP-*Pp*PDK1-6His was added to 10 µl of Ni_{2+} resin (Novagen) in 200 µl of binding buffer (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 5 mM imidazole, 0.1% Triton- X-100) and mixed at 4°C for 1 hr to allow MBP-*Pp*PDK1-6His to bind the resin. Next, 5 µg of purified MBP or MBP-*Pp*2411 was added to the sample and mixed at 4°C for 1 hr to allow the proteins to interact with MBP-*Pp*PDK1-6His. After the incubation period samples were centrifuged at 400 x *g* for 1 min, the supernatant removed, and the samples were washed 3 times with 1 ml binding buffer followed by the addition of 100 µl of 4x SDS-PAGE sample buffer. Ten µl of each sample was then separated by SDS-PAGE and MBP fusion proteins detected by α- MBP western blot. Protein inputs were analyzed by performing SDS-PAGE on 5 µg of purified MBP, MBP-*Pp*2411, and MBP-*Pp*PDK1-6His and staining with coomassie. Binding of MBP, MBP-*At*PDK1-6His, MBP-*Sl*PDK1-6His, and MBP-*Pp*PDK1-6His to several common phospholipids and sphingolipids was tested by protein-lipid overlay using PIP Strips and Sphingo Strips (Echelon Bioscience) according to the manufacturer's instructions and as previously described (Zegzouti et al., 2006). Briefly, lipid strips were blocked with 3% fatty acid-free BSA in 1x PBS for 16 hr, 5 µg of each purified protein was incubated with a lipid strip for 1.5 hr at room temperature, the lipid strips were washed 3 times with 1x PBST, and proteins bound to lipids were detected using α-MBP as with typical western blots.

2.5 Tissue culture of *P. patens* (Chapter III)

Wild type *P. patens* Gransden (Ashton and Cove, 1977) was routinely grown on BCD plates overlaid with cellophane disks (AA Packaging) at 25℃ in continuous light following standard protocols (Roberts et al., 2011). Seven days before transformation, wild type *P. patens* was passaged to BCD plates containing 5 mM di-ammonium tartrate. For each moss stress treatment, all 4 moss lines (wild-type, *pdk1* knockout, *PpPDK1-6His*, and *PpPDK1*^{K71A}*-6His*) were grown on the same plate to ensure results were not due to variation between plates. For heat stress, moss lines were moved to BCD plates and incubated at 30°C for 14 days, then allowed to recover on the same plate at 25°C for 14 days. For osmotic stress, moss lines were moved to BCD plates containing 0.9 M mannitol for 14 days, then moved to new BCD plates lacking mannitol and allowed to recover for 14 days.

2.6 Generation of *pdk1* knockout and *PpPDK1* gene replacement constructs (Chapter III)

For both knockout and gene replacement generation, the pBHRF and pBNRF vectors were used, respectively. In short, pBNRF and pBHRF contain *35S:NptII* (G418/kanamycin resistance) or *35S:Hph* (hygromycin resistance) *Eco*RI fragments from pHP23b or pGL2, respectively, cloned into the *Eco*RI site of pBilox, a derivative of pMCS5 (MoBiTec), with two direct repeats of the loxP sites cloned into the *Xho*I-*Kpn*I and *Bgl*II/*Spe*I sites (Schaefer et al., 2010).

The construct used to generate the *pdk1* knockout line was made by amplifying a 1,091 nt 5’ targeting fragment from genomic DNA with primers 5’-

GAC**AAGCTTC**TCAGAAGTGCAAAGGCTTTCATTC-3' (*Hind*III restriction site in bold) and 5'-GAC**CTCGAG**TCCTGTAATGTTGGTGCC-3' (*Xho*I restriction site in bold) and cloned into the *Hind*III and *Xho*I sites of the vector pBHRF (Schaefer et al., 2010) upstream of *Hph*. Similarly, a 1,151 nt 3' targeting fragment was amplified from genomic DNA with primers 5'-GAC**GCGGCCGC**ATTATCAGTAGACTCACATG-3' (*Not*I restriction site in bold) and 5'-GAC**AGATCT**GATATGTAACACCAACCTAG-3' (*Bgl*II restriction site in bold) and cloned into the *Not*I and *Bgl*II sites of pBHRF, 3' to *Hph*.

The construct used to replace the endogenous *PpPDK1* locus with *PpPDK1-6His* or *PpPDK1*^{K71A}*-6His* was made by amplifying a 1,091 nt 5' targeting fragment plus the entire *PpPDK1* coding region from genomic DNA with primers 5'-AAGTGCAAAGGCT TTCAATC-3' and 5'-TACGTCGTATACAAATGCATCC-3' and cloning into pCR2.1-TOPO (Invitrogen). A C-terminal 6His tag, a 5' *Xba*I site, and a 3' *Xho*I site were added to this *PpPDK1* by PCR and cloned into the *Xba*I and *Xho*I sites of pBNRF upstream of *NptII*. Similarly, a 1,151 nt 3' targeting fragment was amplified from genomic DNA with primers 5'-GAC**GCGGCCGC**ATTATCAGTAGACTCACATG-3' (*Not*I restriction site in bold) and 5'-GAC**AGATCT**GATATGTAACACCAACCTAG-3' (*Bgl*II restriction site in bold) and cloned into the *Not*I and *Bgl*II sites of pBNRF, 3' to *NptII*. The K71A mutation was then introduced by site-directed mutagenesis using Pfu Turbo according to the manufacturer's instructions

The construct used to replace the endogenous *PpPDK1* locus with *PpPDK1-GFP* or *PpPDK1*^{K71A}*-GFP* was made by amplifying a 1,091 nt 5' targeting fragment plus the entire *PpPDK1* coding region from genomic DNA with primers 5'-GAC**CCCGGG**AAGTGCAAAGGCT TTCAATC-3' (*Sma*I restriction site in bold) and 5'-GAC**CTCGAG**TACGTCGTATACAAATGCATCC-3' (*Xba*I restriction site in bold) and cloning into pTEX to add a C-terminal GFP tag. Either a 5' *Sma*I site and a 3' *Xho*I site (for cloning into pBNRF) or a 5' AvrII site and a 3' XhoI site (for cloning into pBHRF) were added to this *PpPDK1-GFP* by PCR and cloned into the *Sma*I and *Xho*I sites of pBNRF upstream of *NptII* or the *Avr*II and *Xho*I sites of the vector pBHRF upstream of *Hph*. Similarly, a 1,151 nt 3' targeting fragment was amplified from genomic DNA with primers 5'- GAC**GCGGCCGC**ATTATCAGTAGACTCACATG-3' (*Not*I restriction site in bold) and 5'-GAC**AGATCT**GATATGTAACACCAACCTAG-3' (*Bgl*II restriction site in bold) and cloned into the *Not*I and *Bgl*II sites of pBNRF or pBHRF, 3' to *NptII* or *Hph*. The final cloning step consisted of amplifying the nopaline synthase (NOS) terminator with primers 5'-GAC**CTCGAG**GATCGTTCAAACATTTGG-3' and 5'-GAC**CTCGAG**ATAGATGACACCGCG-3' (*Xho*I sites in bold) and cloning it into the *Xho*I site of pBNRF or pBHRF to produce the final construct. The K71A mutation was then introduced by site-directed mutagenesis using Pfu Turbo according to the manufacturer's instructions.

To transform *P. patens*, 50-100 μg of each construct was linearized by simultaneous digestion with 80 units of *Hind*III and *Spe*I for *pdk1* knockout generation,

*Xba*I and *Spe*I for *PpPDK1-6His* gene replacement, or either *Sma*I and *Spe*I or *Avr*II and *Spe*I for *PpPDK1-GFP* gene replacement. Linearized DNA was introduced into wild type *P. patens* Gransden protoplasts using a previously described PEG transformation protocol (Roberts et al., 2011). Transformants were initially selected by 7 days of growth on plates containing 20 μg/ml hygromycin or 25 μg/ml G418 followed by 10 days of growth on nonselective plates and an additional 7 days of growth on hygromycin or G418 plates. Surviving colonies were analyzed by genotyping PCR and Southern blotting for integration of the *Pp*PDK1-6His constructs into the correct location in the genome.

2.7 Genomic DNA extraction (Chapter III)

Genomic DNA was extracted from each moss colony surviving two rounds of hygromycin or G418 selection by grinding the tissue (approximately 10-25 mm^2 per colony) in a microcentrifuge tube with 200 μl extraction buffer (2% hexadecyltrimethylammonium bromide (CTAB), 100 mM Tris pH 8.0, 20 mM EDTA, 1.4 M NaCl) followed by incubation at 65°C for 30 min. An equal volume (200 μl) of phenol/chloroform was added and the mixture was vortexed vigorously for 15 sec followed by centrifugation at 15,000 x *g* for 15 min. The upper aqueous phase was added to a new microcentrifuge tube containing 170 μl isopropanol and 10 μl of 3 M sodium acetate, pH 5.2. Samples were incubated at -20°C for 1 hr followed by centrifugation at 15,000 x *g* for 20 min. The supernatant was aspirated and pellets washed with 200 μl of 70% ethanol followed by centrifugation at 15,000 x *g* for 20 min. Pellets were dried for

1 hr at room temperature and resuspended at 37°C in 20 µl of autoclaved dH_2O containing 10 µg RNase A.

2.8 Genotyping *P. patens* (Chapter III)

Genomic DNA extracted as described above was subjected to genotyping PCR. Initial genotyping was performed with primers that amplify a 532 nt product if the endogenous PpPDK1 is present, a 2,356 nt product if a PpPDK1-6His construct is present, and no product if the endogenous PpPDK1 is not present; primer 1, 5'-GAGAGACTGGGAGTTCAAGGCTATG-3' and primer 2, 5'-CTCCAACTGGTAATTAATGCTGCAATGG-3'. A diagram of the locations of these genotyping primers can be found in Fig. 5A and B.

Additional genotyping PCR was used to test for proper 5' and 3' integration of the pdk1 knockout and PpPDK1-6His constructs. The first set of primers tests for 5' integration. These primers are based outside the 5' targeting fragment (primer 3) and in the 35S promoter (primer 4) and amplify the entire 5' targeting fragment for the pdk1 knockout construct and the entire 5' targeting fragment plus the PpPDK1-6His coding sequence for the gene replacement constructs; primer 3, 5'-GGTAGGTGGTATTTCTAACACTCAATGATG-3' and primer 4, 5'-CGTGCTCCACCATGTTGACGAAG-3'. The second set of primers tests for 3' integration. These primers are based in the *NptII* gene (primer 5) and outside the 3' targeting fragment (primer 6) and amplify the entire 3' targeting fragment for all constructs; primer 5, 5'-GCTGAAATCACCAGTCTCTCTCTAC-3' and primer 6, 5'-GGCAATGGTTCAAAAACCTCTTATAAGTCC-3'.

2.9 Southern blot (Chapter III)

Southern blot analysis was performed essentially as previously described (Nelson et al., 2011) to verify that *PpPDK1-6His* or *PpPDK1*^{K71A}*-6His* was integrated into the correct location in the *P. patens* genome and to assess the number of integration events. Forty μg of genomic DNA extracted from wild-type, *PpPDK1-6His*, or *PpPDK1*^{K71A}*-6His* moss was simultaneously digested with 120 units each of *Nde*I, *Sal*I, and *Xba*I and separated on a 1% agarose gel, which was then transferred to a nylon membrane (GE Healthcare). The membrane was hybridized at 65°C with a High Prime (Roche) internally labeled DNA probe comprised of 234 nt of the 35S promoter used to drive *NptII* expression in pBNRF. This probe was amplified from the pBNRF vector using primers 5'-CGTCAACATGGTGGAGCACG-3' and 5'-GCAGAGGCATCTTCAACGATGG-3'.

2.10 Expression analysis of *PpPDK1* in transformed moss (Chapter III)

Total RNA was extracted from *P. patens* tissue using TRIzol Reagent (Invitrogen) and reverse transcription was performed using qScript cDNA SuperMix (Quanta Biosciences) according to the manufacturer's instructions. RT-PCR was performed as previously described (Harries et al., 2005). As an expression control, ubiquitin was amplified with forward primer 5'-ACTACCCTGAAGTTGTATAGTTCGG-3' and reverse primer 5'-CAAGTCACATTACTTCGCTGTCTAG-3'. *PpPDK1* was amplified with forward primer 5'- TTCAAAGCTGCGACAGAATATTTGAC-3' and reverse primer 5'-CTTGCCATTTTTCTTCTTCATCCAAAC-3'. Each gene was amplified by 30 cycles of

PCR, utilizing a 30 sec denaturing step at 94°C, a 30 sec annealing step at 57°C, and a 30 sec extension step at 72°C.

2.11 Phylogenetic analysis of 23 PDK1 proteins from plants and algae (Chapter IV)

PDK1 protein sequences shown in Table 3 were obtained from various genome databases. A multiple alignment of all 23 PDK1 protein sequences was created using MUSCLE (MUltiple Sequence Comparison by Log-Expectation). A maximum-likelihood phylogenetic tree was created from aligned PDK1 sequences using MEGA5 (Tamura et al., 2011) and the resulting phylogram labeled in Adobe Illustrator.

2.12 Phylogenetic analysis of putative PDK1 proteins from 100 diverse eukaryotic species (Chapter IV)

PDK1 protein sequences shown in Table 4 were obtained from NCBI, Phytozome, JGI, and individual species genome databases. A multiple alignment of all 100 PDK1 protein sequences was created using MUSCLE. A maximum-likelihood phylogenetic tree was then created from aligned PDK1 sequences using MEGA5 (Tamura et al., 2011) and the resulting phylogram labeled in Adobe Illustrator.

2.13 Cloning and site directed mutagenesis (Chapter V)

Cloning of the PDK1 and Adi3 cDNA into pMAL-c2 and expression/purification of protein from *E. coli* for N-terminal maltose binding protein (MBP) translational fusions was previously described (Devarenne et al., 2006). Site-directed mutagenesis was carried out using standard protocols and Pfu Turbo DNA polymerase (Stratagene). The Adi3 S539D and K337Q mutations were previously described (Devarenne et al., 2006). Primers for the S212A and S212D mutations are as follows (mutation sites in

Table 3. Putative PDK1 sequences from 18 species of plants and algae. These proteins were used in the Archaeplastida PDK1 phylogeny in Chapter IV.

Putative PDK1s used in phylogeny Organism	Accession number	Website where PDK1 was obtained	Amino acids in protein	Lipid-binding?
Populus trichocarpa PDK1-1	XP_002315349.1	NCBI: http://www.ncbi.nlm.nih.gov/	496	PDK1-like PH
Populus trichocarpa PDK1-2	POPTR_0008s02890.1	Phytozome: http://www.phytozome.net/	444	PDK1-like PH
Ricinus communis PDK1	XP_002533941.1	NCBI	506	PDK1-like PH
Glycine max PDK1-1	XP_002262670.1	NCBI	491	PDK1-like PH
Glycine max PDK1-2	Glyma20g33140	Phytozome	491	PDK1-like PH
Vitis vinifera PDK1	CBI40191.3	NCBI	468	PDK1-like PH
Arabidopsis thaliana PDK1-1	AED90755.1	NCBI	491	PDK1-like PH
Arabidopsis thaliana PDK1-2	NP_187665.2	NCBI	486	PDK1-like PH
Solanum lycopersicum PDK1-1	AAW38936.1	NCBI	494	PDK1-like PH
Solanum lycopersicum PDK1-2	SL1.00sc03032_9.1.1	Tomato genome: http://solgenomics.net	480	PDK1-like PH
Oryza sativa PDK1	BAF06862.1	NCBI	498	PDK1-like PH
Sorghum bicolor PDK1	XP_002458841.1	NCBI	504	PDK1-like PH
Zea mays PDK1-1	ABB71956.1	NCBI	495	PDK1-like PH
Zea mays PDK1-2	ACG46841.1	NCBI	504	PDK1-like PH
Selaginella moellendorffii PDK1	XP_002960408.1	NCBI	489	PH-like
Physcomitrella patens PDK1	JN049607	NCBI	347	Not detected
Chlorella sp. NC64A PDK1	26267	JGI: http://www.jgi.doe.gov/	317	Not detected
Chlamydomonas reinhardtii PDK1	XP_001701378.1	NCBI	343	Not detected
Volvox carteri PDK1	XP_002957069.1	NCBI	340	Not detected
Ostreococcus lucimariinus PDK1	14571	JGI	682	Not detected
Ostreococcus tauri PDK1	XP_003078129.1	NCBI	804	Not detected
Cyanidioschyzon merolae PDK1	CMO090C	C. merolae genome: http://merolae.biol.s.u-tokyo.ac.jp/	712	Not detected
Ectocarpus siliculosus PDK1	CBJ33478	NCBI	756	Not detected

Table 4. Putative PDK1 sequences from 100 species of eukaryotes. These proteins were used in the eukaryote PDK1 phylogeny in Chapter IV.

Putative PDK1s used in phylogeny Organism	Accession number	Website where PDK1 was obtained	Amino acids in protein	Lipid-binding?
Bos taurus	XP_001788715	NCBI: http://www.ncbi.nlm.nih.gov/	754	PDK1-like PH
Canis lupus familiaris	XP_537000.2	NCBI	560	PDK1-like PH
Homo sapiens	NP_002604.1	NCBI	556	PDK1-like PH
Monodelphis domestica	XP_001375220.1	NCBI	573	PH-like
Gallus gallus	NP_001012547	NCBI	556	PDK1-like PH
Taeniopygia guttata	XP_002193533	NCBI	524	PDK1-like PH
Anolis carolinensis	XP_003228307.1	NCBI	546	PDK1-like PH
Danio rerio	NP_991262	NCBI	537	PDK1-like PH
Dicentrarchus labrax	CBN81295.1	NCBI	564	PDK1-like PH
Xenopus laevis	NP_001086100	NCBI	506	PDK1-like PH
Xenopus tropicalis	XP_002938460	NCBI	796	PDK1-like PH
Hydra magnipapillata	ACO52516.1	NCBI	457	PDK1-like PH
Patiria pectinifera	BAD02370	NCBI	571	PDK1-like PH
Strongylocentrotus purpuratus	XP_001189048	NCBI	539	PDK1-like PH
Aplysia californica	NP_001191464.1	NCBI	822	PDK1-like PH
Caenorhabditis briggsae	XP_002643197.1	NCBI	630	PDK1-like PH
Caenorhabditis elegans	CCD67851.1	NCBI	636	PDK1-like PH
Branchiostoma floridae	XP_002606093.1	NCBI	474	Not detected
Tribolium castaneum	EFA00152.1	NCBI	475	PDK1-like PH
Daphnia pulex	EFX72775.1	NCBI	512	PDK1-like PH
Drosophila melanogaster	NP_728471.2	NCBI	836	PDK1-like PH
Haemaphysalis longicornis	BAK64411.1	NCBI	564	PDK1-like PH
Acromyrmex echinatior	EGI66916	NCBI	550	PDK1-like PH
Acyrthosiphon pisum	XP_001951019.2	NCBI	617	PDK1-like PH
Trichoplax adhaerens	XP_002116894.1	NCBI	462	PDK1-like PH

Table 4, continued.

Putative PDK1s used in phylogeny Organism	Accession number	Website where PDK1 was obtained	Amino acids in protein	Lipid-binding?
Amphimedon queenslandica	XP_003382943.1	NCBI	478	PDK1-like PH
Salpingoeca sp. ATC50818	EGD83036.1	NCBI	516	PDK1-like PH
Monosiga brevicollis	XP_001750395.1	NCBI	239	Not detected
Gonapodya prolifera	235740	JGI: http://www.jgi.doe.gov/	333	Not detected
Phytophthora infestans	XP_002909566.1	NCBI	485	PH-like
Phytophthora ramorum	82481	JGI	545	PH-like
Trichomonas vaginalis	XP_001583554.1	NCBI	472	PH-like
Sporobolomyces roseus	28831	JGI	1987	Not detected
Puccinia graminis	XP_003323291.1	NCBI	830	Very weak, partial PH-like
Batrachochytrium dendrobatidis	BDEG_00769	Broad Institute: http://www.broadinstitute.org/	387	Not detected
Schizosaccharomyces pombe	CAA21194.1	NCBI	592	PDK1-like PH
Mucor circinelloides	159810	JGI	575	PH-like
Rhizopus oryzae	3800	JGI	337	Not detected
Cryptococcus neoformans	XP_570023.1	NCBI	1206	Not detected
Piriformospora indica	CCA68012.1	NCBI	953	Not detected
Malassezia globosa	XP_001731977.1	NCBI	801	Very weak, partial PH-like
Ustilago maydis	XP_761169.1	NCBI	1667	Very weak, partial PH-like
Saccharomyces cerevisiae	NP_010778	NCBI	766	Not detected
Glomus intraradices	step3_c2197	GlomusDB: http://mycor.nancy.inra.fr/IMGC/GlomusGenome/	484	PH-like
Cladonia grayi	99595	JGI	910	Not detected
Xanthoria parietina	49490	JGI	875	Not detected
Arthroderma otae	EEQ27454.1	NCBI	813	Not detected
Aspergillus terreus	EAU35932.1	NCBI	593	Not detected
Neurospora crassa	CAD70304.1	NCBI	922	Not detected
Saitoella complicata	69909	JGI	879	Very weak, partial PH-like

Table 4, continued.

Putative PDK1s used in phylogeny Organism	Accession number	Website where PDK1 was obtained	Amino acids in protein	Lipid-binding?
Dictyostelium discoideum	XP_640699.1	NCBI	686	PH-like
Dictyostelium purpureum	XP_003290409.1	NCBI	506	PH-like
Entamoeba histolytica	XP_654883.1	NCBI	395	PH-like
Naegleria gruberi	EFC39666	NCBI	398	PH-like
Ciona intestinalis	XP_002128872	NCBI	476	PH-like
Paramecium tetraurelia	XP_001435296.1	NCBI	445	Not detected
Tetrahymena thermophila	XP_001016569.2	NCBI	536	PH-like
Ostreococcus tauri	XP_003078129.1	NCBI	804	Not detected
Ostreococcus lucimarinus	14571	JGI	682	Not detected
Micromonas sp. RCC299	XP_002512313.1	NCBI	329	Not detected
Chlorella sp. NC64A	26267	JGI	317	Not detected
Volvox carteri	XP_002957069.1	NCBI	340	Not detected
Chlamydomonas reinhardtii	XP_001701378.1	NCBI	343	Not detected
Physcomitrella patens	JN049607	NCBI	347	Not detected
Selaginella moellendorffii	XP_002960408.1	NCBI	489	PH-like
Sorghum bicolor	XP_002458841.1	NCBI	504	PDK1-like PH
Zea mays	ABB71956.1	NCBI	495	PDK1-like PH
Brachypodium distachyon	XP_003564737.1	NCBI	503	PDK1-like PH
Hordeum vulgare	BAJ94328.1	NCBI	501	PDK1-like PH
Oryza sativa	BAF06862.1	NCBI	498	PDK1-like PH
Setaria italica	Si001110m.g	Phytozome: http://www.phytozome.net/	508	PDK1-like PH
Pinus taeda	isotig38700	Dendrome: http://dendrome.ucdavis.edu/	513	PDK1-like PH
Aquilegia coerulea	Aquca_019_00085	Phytozome	517	PH-like
Eucalyptus grandis	Eucgr.J01029	Phytozome	493	PDK1-like PH
Mimulus guttatus	mgv1a004635m.g	Phytozome	517	PDK1-like PH

Table 4, continued.

Putative PDK1s used in phylogeny Organism	Accession number	Website where PDK1 was obtained	Amino acids in protein	Lipid-binding?
Vitis vinifera	CBI40191.3	NCBI	468	PDK1-like PH
Arabidopsis thaliana	AED90755	NCBI	491	PDK1-like PH
Cucumis sativus	Cucsa.308880	Phytozome	489	PDK1-like PH
Ricinus communis	XP_002533941.1	NCBI	506	PDK1-like PH
Solanum lycopersicum	AAW38936.1	NCBI	494	PDK1-like PH
Citrus sinensis	orange1.1g010859m.g	Phytozome	499	PDK1-like PH
Prunus persica	ppa005175m.g	Phytozome	473	PDK1-like PH
Populus trichocarpa	XP_002315349.1	NCBI	496	PDK1-like PH
Glycine max	XP_002262670.1	NCBI	491	PDK1-like PH
Phaseolus vulgaris	Phvulv091027525m.g	Phytozome	491	PDK1-like PH
Clonorchis sinensis	GAA43227.2	NCBI	623	PDK1-like PH
Schistosoma mansoni	XP_002580355.1	NCBI	484	PDK1-like PH
Coprinopsis cinerea	XP_002910557.1	NCBI	346	Not detected
Laccaria bicolor	629674	JGI	350	Not detected
Leishmania major	XP_001682075.1	NCBI	386	FYVE
Trypanosoma brucei	XP_803794.1	NCBI	384	FYVE
Bigelowiella natans	68079	JGI	317	Not detected
Cyanidioschyzon merolae	CMO090C	C. merolae genome: http://merolae.biol.s.u-tokyo.ac.jp/	712	Not detected
Emiliana huxleyi	284603	JGI	510	Not detected
Plasmodium vivax	XP_001615336	NCBI	561	Not detected
Ectocarpus siliculosus	CBJ33478	NCBI	756	Not detected
Phaodactylum tricornutum	33668	JGI	872	Not detected
Thalassiosira pseudonana	4902	JGI	942	Not detected
Fragilariopsis cylindrus	185182	JGI	810	Not detected
Perkinsus marinus	XP_002780932	NCBI	1106	Not detected

bold): For S212A, forward primer 5'-GTTGTGAGATCTATG**GCA**ATTGTCAACAGTTGC-3', reverse primer 5'-GCAACTGTTGACAAT**TGC**CATAGATCTCACAAC-3'; for S212D, forward primer 5'-GTTGTGAGATCTATG**GAC**ATTGTCAACAGTTGC-3', reverse primer 5'-GCAACTGTTGACAAT**GTC**CATAGATCTCACAAC-3'.

2.14 In vitro kinase assays (Chapter V)

PDK1, Adi3, and Gal83 were expressed as MBP fusion proteins using pMAL-c2 in *E. coli* BL21(DE3) and purified with amylose resin (New England Biolabs). In vitro kinase assays were performed by combining the purified proteins in a 30 µl final volume of kinase buffer containing 10 mM Tris pH 7.5, 10 mM $MgCl_2$ or 10 mM $MnCl_2$, and 1 mM DTT. PDK1 phosphorylation of Adi3 and Adi3 autophosphorylation kinase assays were carried out with 0.5 µg of purified MBP-PDK1 protein, 5 µg of purified MBP-Adi3 protein, and 0.25 µCi of γ-[^{32}P]ATP as previously described (Devarenne et al., 2006). Adi3 phosphorylation of Gal83 kinase assays were carried out with 0.2 µg of purified MBP-PDK1 protein, 0.4 µg of purified MBP-Adi3 protein, 2 µg of purified MBP-Gal83 protein, and 0.25 µCi of γ-[^{32}P]ATP. The reactions were started by the addition of 0.25 µCi γ-[^{32}P]ATP and nonradiolabeled ATP to a final concentration of 20 µM followed by incubation at room temperature for 15 min. Reactions were stopped by the addition of 4x SDS-PAGE sample buffer. Protein phosphorylation was visualized by phosphorimager (Bio-Rad Molecular Imager) after separation by SDS-PAGE and signals were quantified using Quantity One software (Bio-Rad). All kinase assays were performed a minimum of three times, with representative images shown in figures.

2.15 Cloning and site directed mutagenesis (Chapter VI)

Cloning of the *Adi3* cDNA into pMAL-c2 and expression/purification of protein from *E. coli* for N- terminal maltose binding protein (MBP) translational fusions was previously described (Devarenne et al., 2006). Site-directed mutagenesis was carried out using standard protocols and Pfu Turbo DNA polymerase (Stratagene). The Adi3 S539D and K337Q mutations were previously described (Devarenne et al., 2006). Primers for the M385A and M385G mutations are as follows (mutation sites in bold): For M385A, forward primer 5'-TTCTCATGTTTGGTC**GCA**GAAATATTGTCCTGGA-3', reverse primer 5'-TCCAGGACAATATTTC**TGC**GACCAAACATGAGAA-3'; for M385G, forward primer 5'TTCTCATGTTTGGTC**GGA**GAATATTGTC CTGGA-3', reverse primer 5'-TCCAGGACAATATTTC**TCC**GACCAAACATGAGAA-3'. v-Src and v-SrcI338G (Shah et al., 1997) were cloned into pMAL-c2 and expressed and purified from *E. coli* as with MBP-Adi3.

2.16 Production of bulky ATP analogue (Chapter VI)

Gamma radiolabeled N6-benzyl-ATP was produced following previously published protocols (Bohmer and Romeis, 2007; Habelhah et al., 2001; Blethrow et al., 2004). Briefly, nucleoside diphosphate kinase (NDPK) (Amutha and Pain, 2003) was cloned into pET21b to produce *E. coli* expressed C-terminal 6xHis tagged protein, which was purified using Ni^{+2} resin (Nonagon). Eighty μg of NDPK-6xHis was immobilized on a 130 μl Ni^{+2} column followed by addition to the column of 360 pmol (1 mCi at 3000 Ci/mmol) of γ -[^{32}P]ATP diluted in 800 μl of 1x PBS. After washing the column thoroughly with 1x PBS, 8 nmol of non-radiolabeled N6-benzyl-ADP (BioLog Life

Science Institute) was diluted in 32 µl of 1x PBS containing 5 mM MgCl2, added to the column, and incubated at room temp for 5 min. Finally, γ -[^{32}P]-N6-benzyl-ATP was eluted from the column with a buffer of 100 µl 1x PBS, 5 mM $MgCl_2$ and stored in 10 µl aliquots at -80°C until used.

2.17 In vitro kinase assays (Chapter VI)

Adi3 autophosphorylation kinase assays were carried out with 5 µg of purified MBP-Adi3 protein and 0.25 µCi of γ-[^{32}P]ATP or γ-[^{32}P]-N6-benzyl-ATP per reaction in a volume of 30 µl as previously described (Devarenne et al., 2006). Reactions were stopped by addition of 4x SDS-PAGE sample buffer and samples separated by 10% SDS-PAGE followed by visualization and quantification of incorporated radioactivity using a phosphorimager (Bio-Rad Molecular Imager) and quantification software (Bio-Rad Quantity One). Adi3 phosphorylation of Gal83 was carried as above with 1 µg MBP-Adi3 protein and 5 µg of MBP-Gal83 as previously described. All kinase assays were carried out two or three independent times. Values shown for phosphorylation levels are from one experiment and are representative of all experiments. v-Src kinase assays were carried out with 3 µg of purified MPB-vSrc protein under conditions previously described (Shah et al., 1997).

2.18 Adi3 phosphorylation of tomato protoplast lysate in a creatine kinase ATP-regenerating buffer (Chapter VI)

Starting material for this experiment was either 2 µg purified MBP-Adi3 or the lysate from 300,000 protoplasts in a buffer containing 10 mM Tris pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 1x plant protease inhibitor cocktail, 20 mM phosphocreatine, 50

μg/ml creatine kinase, 100 μM MG-132, and 0.5% Triton-X-100. Reactions were started with the addition of 1x plant phosphatase inhibitor cocktail, non-radiolabeled ATP to 1 mM, 1 μCi γ-[^{32}P]-N6-benzyl-ATP per reaction in a volume of 60 μl (for purified proteins) or 100 μl (for protoplast lysate). Reactions were incubated at room temperature for 15 minutes and stopped by addition of 4x SDS-PAGE sample buffer and samples separated by 10% SDS-PAGE followed by visualization and quantification of incorporated radioactivity using a phosphorimager (Bio-Rad Molecular Imager) and quantification software (Bio-Rad Quantity One).

2.19 Adi3 phosphorylation of tomato protoplast lysate in a buffer with ATPγS (Chapter VI)

Starting material for this experiment was either 1 μg purified MBP-Adi3 or the lysate from 300,000 protoplasts in a buffer containing 10 mM Tris pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 1x plant phosphatase inhibitor cocktail, 10 μM MG-132, and 0.5% Triton-X-100. Reactions were started with the addition of 1x plant protease inhibitor cocktail, non-radiolabeled ATPγS to 2 mM, and 1 μCi γ-[^{32}P]-N6-benzyl-ATP per reaction in a volume of 60 μl (for purified proteins) or 100 μl (for protoplast lysate). Reactions were incubated at room temperature for 10 minutes and stopped by addition of 4x SDS-PAGE sample buffer and samples separated by 10% SDS-PAGE followed by visualization and quantification of incorporated radioactivity using a phosphorimager (Bio-Rad Molecular Imager) and quantification software (Bio-Rad Quantity One).

2.20 Adi3 phosphorylation of recombinant Gal83 in tomato protoplast lysate (Chapter VI)

Starting material for this experiment was either 1 μg purified MBP-Adi3 and 3 μg purified MBP-Gal83 or the lysate from 300,000 protoplasts in a buffer containing 10 mM Tris pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 1x plant protease inhibitor cocktail, 100 μM MG-132, and 0.5% Triton-X-100. Reactions were started with the addition of 1x plant phosphatase inhibitor cocktail, non-radiolabeled ATP to 1 mM, 1 μCi γ-[^{32}P]-N6-benzyl-ATP per reaction in a volume of 60 μl (for purified proteins) or 100 μl (for protoplast lysate). Reactions were incubated at room temperature for 15 minutes and stopped by addition of 4x SDS-PAGE sample buffer and samples separated by 10% SDS-PAGE followed by visualization and quantification of incorporated radioactivity using a phosphorimager (Bio-Rad Molecular Imager) and quantification software (Bio-Rad Quantity One).

CHAPTER III

CHARACTERIZATION OF A MOSS PDK1 HOMOLOGUE*

3.1 Rationale

In this chapter I present studies investigating a PDK1 homologue from the moss *P. patens*, one of the most primitive extant land plants. This is the first report of an AGC kinase in a non-flowering plant. Because mosses, liverworts, and hornworts diverged early in plant evolution (Fig. 10), studying PDK1s from *P. patens* and other bryophytes will help us understand how functional and regulatory features of plant PDK1s evolved.

3.2 Identification of a putative PDK1 from *P. patens*

3.2a Identification of PDK1 in the P. patens genome

The *P. patens* genome database at Phytozome (www.phytozome.net) was searched using the *A. thaliana* PDK1 (*AtPDK1*) sequence (GenBank accession AF132742.1) (Deak et al., 1999). The two top hits, *Pp1s217_11V6.2* and *Pp1s217_11V6.1* (Fig. 11), are alternate transcripts produced from the same genomic locus, *Pp1s217_11V6*, which spans 3,941 nt of genomic DNA and contains a 2,803 nt coding region of nine exons/eight introns (Fig. 12A; Fig. 13A). *Pp1s217_11V6.2* is listed as the primary transcript, and thus is the main focus of this study. Its coding sequence comprises 1,044 nt, which produces a protein of 347 a.a. (Fig. 12, A and B; Fig. 13, B and C).

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*Portions of the following article have been reprinted with permission: **Dittrich ACN, Devarenne TP** (2012) Characterization of a PDK1 homologue from the moss Physcomitrella patens. Plant Physiology **158:** 1018-1033.

A

BLAST AtPDK1-1 protein sequence to P. patens proteome at Phytozome, top hits:

1. Pp1s217_11V6.2 (Phypa_144576), score 369.0, E value 3.0e-102 (1044 nt PpPDK1 transcript)

2. Pp1s217_11V6.1 (Phypa_144576), score 370.5, E value 1.2e-102 (1041 nt PpPDK1 transcript)

3. Pp1s118_230V6 (Phypa_188412), score 224.2, E value 1.2e-58. Annotated as ribosomal S6 protein kinase and related proteins. BLAST of the cDNA below to TAIR10 cDNAs returned AT3G08730.1 (S6K), AT3G08720.2 (S6K2), and AT3G08720.1 (S6K2) as the top hits

4. Pp1s4_386V6.1 (Phypa_174181), score 223.8, E value 1.7e-57. Annotated as ribosomal S6 protein kinase and related proteins. BLAST of the cDNA below to TAIR10 cDNAs returned AT3G08720.2 (S6K2), AT3G08730.1 (S6K), and AT3G08720.1 (S6K2) as the top hits

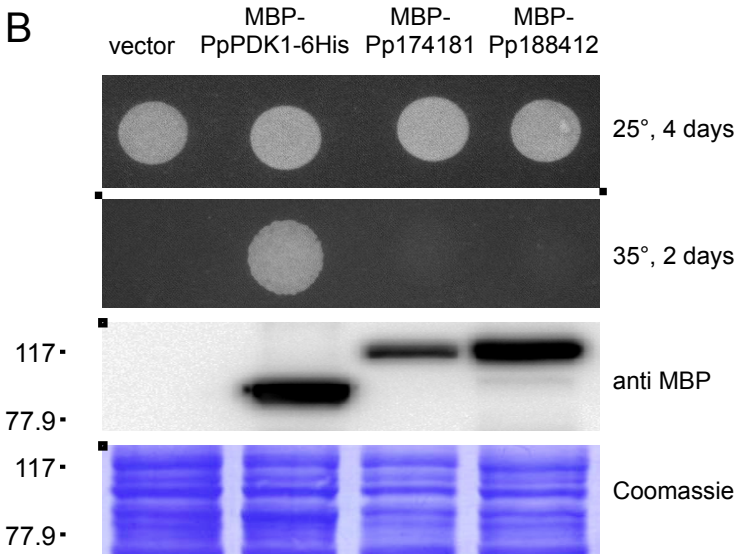

Figure 11. Evidence for one *PDK1* in the *P. patens* genome. A, The *At*PDK1-1 protein sequence (GenBank accession AF132742.1) was used in a BLAST search of the *P. patens* proteome at Phytozome (www.phytozome.net). The names of the top 4 hits, as well as the score and E value for each, are ranked by E value. The first two hits are alternately spliced transcripts from the same *PDK1* locus. The first hit is *Pp1s217_11V6.2*, the 1044 nt *PpPDK1* transcript characterized in this manuscript. The second hit is *Pp1s217_11V6.1*, a 1041 nt *PpPDK1* transcript identical to *Pp1s217_11V6.2* except that it lacks a single glutamate from the splice junction between exons 5 and 6. The third hit is *Pp1s118_230V6* (*Pp188412*), a putative ribosomal S6 kinase. The fourth hit is *Pp1s4_38V6.1* (*Pp174181*), another putative ribosomal S6 kinase, suggesting that the *P. patens* genome contains only one *PDK1*. The cDNA sequences of *Pp188412* and *Pp174181* were used in a BLAST search of *A. thaliana* cDNAs at The Arabidopsis Information Resource (TAIR, www.arabidopsis.org). The names of the top 3 hits are also shown. The top three hits in both cases were *AT3G08730.1* (S6K), *AT3G08720.2* (S6K2), and *AT3G08720.1* (S6K2). B, *Pp*188412 and *Pp*174181 are not able to complement a temperature sensitive allele of *S. cerevisiae PKH1*. Haploid strain INA106-3B, which lacks *PKH2* and contains temperature-sensitive *PKH1D398G*, was transformed with p416GPD containing the indicated constructs under control of the constitutive GPD promoter. Transformed yeast were grown in liquid medium lacking uracil, spotted on plates lacking uracil, and grown at the indicated temperatures and times. Total protein was extracted from cultures grown in liquid medium at 25°C and analyzed by α-MBP western blot to verify expression of each *Pp*PDK1.

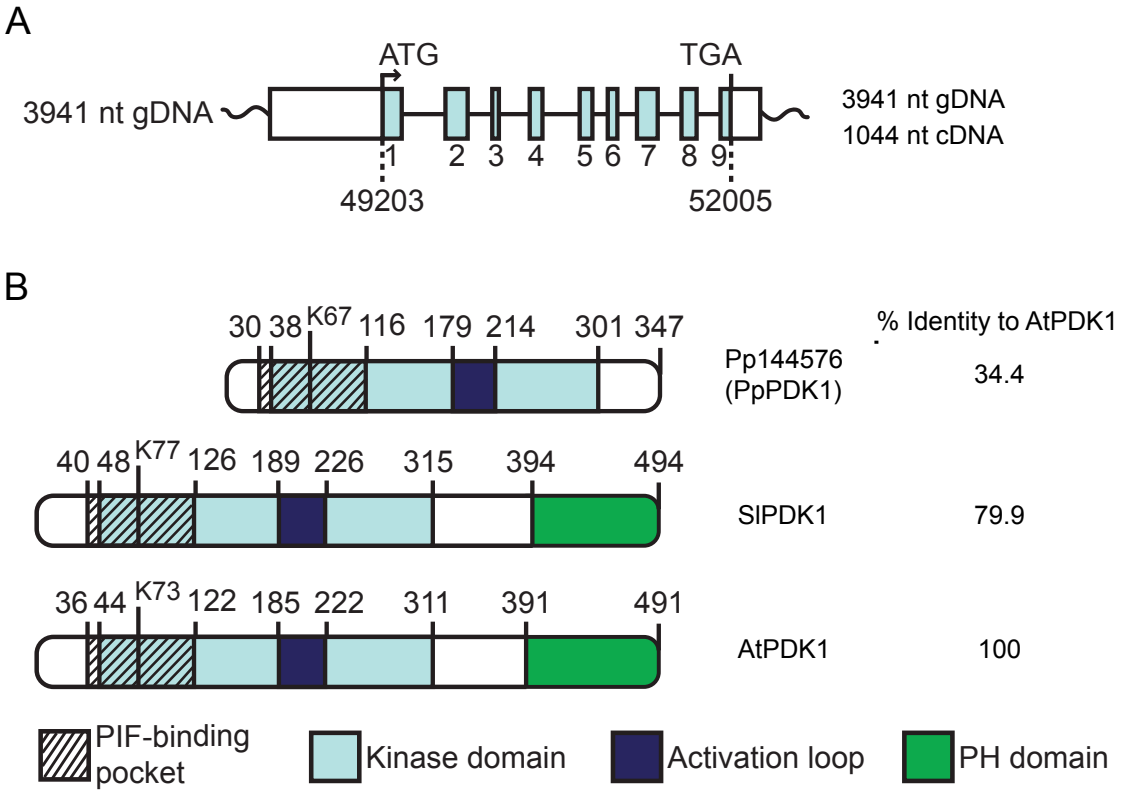

Figure 12. Features of *Pp*PDK1 and comparison with PDK1 from tomato (*Sl*PDK1) and *A. thaliana* (*At*PDK1). A, Diagram of locus *Pp1s217_11V6*, which is located on *P. patens* genomic scaffold 217. The *Pp1s217_11V6* locus spans 3941 nt of genomic DNA consisting of a 901 nt 5' UTR, a 2803 nt coding region of nine exons/eight introns, and a 237 nt 3' UTR. Expression of this gene produces a transcript with 1044 nt of coding sequence. Numbers below the dashed lines indicate the start and end locations of the *PpPDK1* coding sequence on genomic scaffold 217. B, Features of *Pp*PDK1 protein compared with *Sl*PDK1-1 and *At*PDK1-1, as determined by ClustalW multiple alignment of protein sequences. The conserved lysine that is required for Mg2+ coordination in all kinases is K67 in *Pp*PDK1, K77 in *Sl*PDK1, and K73 in *At*PDK1. The percent identity of each protein to *At*PDK1 was determined using the EMBOSS Needle pairwise sequence alignment program.

A

TATAAGTGTTGTCCCACGATTTATATACACTATGGATGTCTCCGTTGTGCATTTCCATGTGTTGATATAAGTTAGAATTGCAATTGATTGA
ATAGGATGTTGAATCTATTGAGATTATTGAGAATGACCACCAAGCCATGATGCGTGCCTACTATAAGGAGATGATTTTCAAAGACATTGTT
GATAAGTGCTCTAATTTGCAAACATAATTTCATGGTGGATGGGCTTATTGTAATGGCCGATTTCCACGACTTCAAATTTTTTGTGGTGGAC
TGGCCTCCATTTTTCCTACCACAAGCATTGTCAAATCCACCTACTTTGTGATGGAGTGGGAGAAGGACGAGTATTGGCAGTGCATCACAAA
CTTCTTGCTTGAGAATATCCTACATTCTAAACAATTCAACAAAGTATGTCATGATTGACAAGGATGGATGTTTTTTGACAACTACATAAAT
GAATTTCATCCATTCCTAATTTACCTTAAAATAAAGTAAATTAGGTATTGCAGGGAAATCCTGGGGACCCAAGCGAGAGCGAGAGAGCAAA
TGCCGGCGAAAACGACCTCGATTGGCGCCCGTCGAGCAGCATCATCGCCCAGACGATGATAGAATCGCGGATTCCTCCGCACAGCCCTGGA
TTGTCAGGGTTGCTCCCCCCTTGTCCTGCTGGAGGCTCGTGATTTTCGTCAGCTTTGCAGCACGCAGCTGCAGTTGAATCGCTGCGTTCTT
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GGGATTAATCGCTGTTTGTCATTGTAAAAGTAGATACTACATTGTGCGTTTTGTATGACGTTGCTTTGTTTAAATGTGTTGAACCTTGGAA
AAAGGTGGGTGTTCTTTTGTACATCGTCCCCATGTTGAATCAGGCATTATGCAATTTTAGGGGATTTTTTTGGATTGTTTCCGAGCGGTTT
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TGGTATTATCAATTTAAGCCATAACATGTGAAAGCTGGCGATGAGCAGGTGACAAAAGCGAAGAGGAAGAACACGGGCGAAATATATGCGT
TGAAGATAATGAACAAGAAGCACATAATTCGTGAGAATAAGGTTAAGTTTGTGAAAATGGAGCGCATGATACTCGACCAGCTCGACCATCC
GGGCGTAGTGAAGCTATGCTTTACATTCCAGGATGTCCACTCTTTGTGTAATGATCATTCTCGCAAATTGTTCTGTGTCTGATCGTCTGCA
CCGCTGTGGATTATTGTCCGTGCAAGTGATCTTAGCTGAGTGCCTGTTAGGCACTTCCAGTACTGAAGAAGACAGACATTTGTTTTAGCCC
ATATGTCCAACCTGTCAATTATGAGCTTGCTACTAATCAGTCTGATTGATGGACATGCTAGACATGGGGCTTGAATGTTGCACTGGTGGAG
AACTTTTCGAGCAGATAAGAAGGGTACGTAATCATATCCAGCAAGAGTACTTGCGCTTCTTTCTCAATTTGGTTTGTATCATATCAAGATT
CTGTCCTAAGCAGCCACTATCAGATCTTCCCAACGGCTCGTCCGGAATTGCTTCGCTTAAATCATTATCCTAAAGTAGATCTTGTTCTTGC
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B

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C

MAMDGTSPVSPEPNQSKPLDPKQLVMRAPQMDFTSNDFLFAKLLGLGSYSKVTKAKRKNTGEIYALKIMNKKHIIRENKVKFVKMERMILD
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LVDSLLNLKPNERLGVQGYDDIKNHPFFKGFDWSRLRKMATPKLLKDPNTESLDEEEKWQAGIIDGLDAFVYDV

Figure 13. *PpPDK1* genomic DNA, cDNA, and protein sequences. A, *PpPDK1* genomic DNA sequence of 3941 nt, with 5' and 3' UTRs shown in blue text, exons in black text, and introns in red text. B, *PpPDK1* coding sequence of 1044 nt. The nucleotide at the 3' end of each exon is in red text. C, *Pp*PDK1 protein sequence of 347 amino acids. K67 is highlighted in green and K71, I75, Q106, and L111 are highlighted in yellow.

Pp1s217_11V6.1 is a splice variant with a 1,041 nt coding sequence generated by including the final 3 nt of exon 5 with intron 5. This produces a protein lacking a single glutamate that would originate from the junction between exons 5 and 6 (data not shown). *Pp1s217_11V6* locus transcripts are annotated as PDK1 by Kyoto Encyclopedia of Genes and Genomes (KEGG). The third and fourth hits (*Pp1s118_230V6* and *Pp1s4_386V6.1*) are annotated as putative homologues of S6K by Eukaryotic Orthologous Groups (KOG) and have coding sequences of 1,488 nt and 1,518 nt, respectively (Fig. 11). *Pp1s217_11V6.2*, *Pp1s118_230V6*, and *Pp1s4_386V6.1* were all amplified from total moss mRNA, cloned into *E. coli* and yeast expression vectors, and sequenced. Hereafter the *Pp1s217_11V6.2*, *Pp1s118_230V6*, and *Pp1s4_386V6.1* genes are referred to as *PpPDK1*, *Pp188412*, and *Pp174181*, respectively (Table 5).

3.2b Features of PpPDK1 protein

Based on ClustalW protein sequence alignment with *At*PDK1 and tomato PDK1 (*Sl*PDK1), *Pp*PDK1 appears to possess characteristics of a typical PDK1: a pocket for binding the PIF motif of substrate AGC kinases, a kinase domain containing the conserved Lys residue (Lys67) required for ATP coordination by all protein kinases, and an activation loop within the kinase domain (Fig. 12B). However, *Pp*PDK1 lacks a C-terminal lipid-binding PH domain, and thus shares only 34.4% amino acid identity with *At*PDK1, whereas *Sl*PDK1 and *At*PDK1 are 79.9% identical (Fig. 12B). Like *Pp*PDK1, yeast Pkh1 and Pkh2 also lack PH domains, but both proteins are much larger than *Pp*PDK1 (Pkh1, 766 a.a.; Pkh2, 1,061 a.a.) and their overall sequence identity to other PDK1s is low (Casamayor et al., 1999).

Table 5. Names of all genes used in Chapter III.

Gene name used in this chapter	GenBank accession ID	Genomic locus at Phytozome	Transcript name at Phytozome	Alias at Phytozome
*At*PDK1	AF132742.1	n/a	n/a	n/a
*Sl*PDK1	AAW38936.1	n/a	n/a	n/a
*Pp*PDK1	JN049607	Pp1s217_11V6	Pp1s217_11V6.2	Phypa_144576
n/a	n/a	Pp1s217_11V6	Pp1s217_11V6.1	Phypa_144576
*Pp*174181	JN049609	Pp1s4_386V6	Pp1s4_386V6.1	Phypa_174181
*Pp*188412	JN049610	Pp1s118_230V6	Pp1s118_230V6.1	Phypa_188412
Pp2484	JN049608	Pp1s224_73V6	Pp1s224_73V6.1	Phypa_2484
Adi3	NP_001234611.1	n/a	n/a	n/a

3.3 *Pp*PDK1 is a functional homologue of *S. cerevisiae* Pkh1/2

3.3a Complementation of Δpkh1/Δpkh2 yeast with plant PDK1s

*At*PDK1 was initially identified as a functional homologue of PDK1 by its ability to complement a deletion strain of the *S. cerevisiae PDK1* homologues *PKH1* and *PKH2* (Deak et al., 1999). The previously characterized diploid yeast strain AC306 is heterozygous for deletion of both *PKH1* and *PKH2* (Casamayor et al., 1999). After sporulation of this strain, haploid cells lacking both *PKH1* and *PKH2* (*pkh1Δ::HIS3*/*pkh2Δ::TRP1*) are inviable, but expression of either human *PDK1* or *AtPDK1* in these cells rescues the lethal phenotype (Casamayor et al., 1999; Deak et al., 1999). A similar complementation experiment was performed to test whether *Pp*PDK1 is a functional homologue of Pkh1/2. AC306 yeast transformed with empty vector, *MBP-AtPDK1-6His*, *MBP-SlPDK1-6His*, or *MBP-PpPDK1-6His* were sporulated and haploid spores grown on nonselective YPD medium to enable growth of all viable spores. Analysis of at least thirty tetrads from each yeast culture recovered no viable *pkh1Δ::HIS3*/*pkh2Δ::TRP1* spores from untransformed yeast or from yeast transformed with empty vector (Fig. 14A).

However, after transformation with *MBP-AtPDK1-6His*, *MBP-SlPDK1-6His*, or *MBP-PpPDK1-6His* viable *pkh1Δ::HIS3*/*pkh2Δ::TRP1* spores were recovered, indicating that each *PDK1* tested was able to rescue the lethal phenotype of the *PKH1/2* deletion (Fig. 14A). Western blotting with α-MBP confirmed expression of the PDK1 proteins (Fig. 14B). The ability of each *PDK1* to confer *pkh1Δ::HIS3*/*pkh2Δ::TRP1* spore viability was confirmed by growing spores on medium with 5- fluoroorotic acid

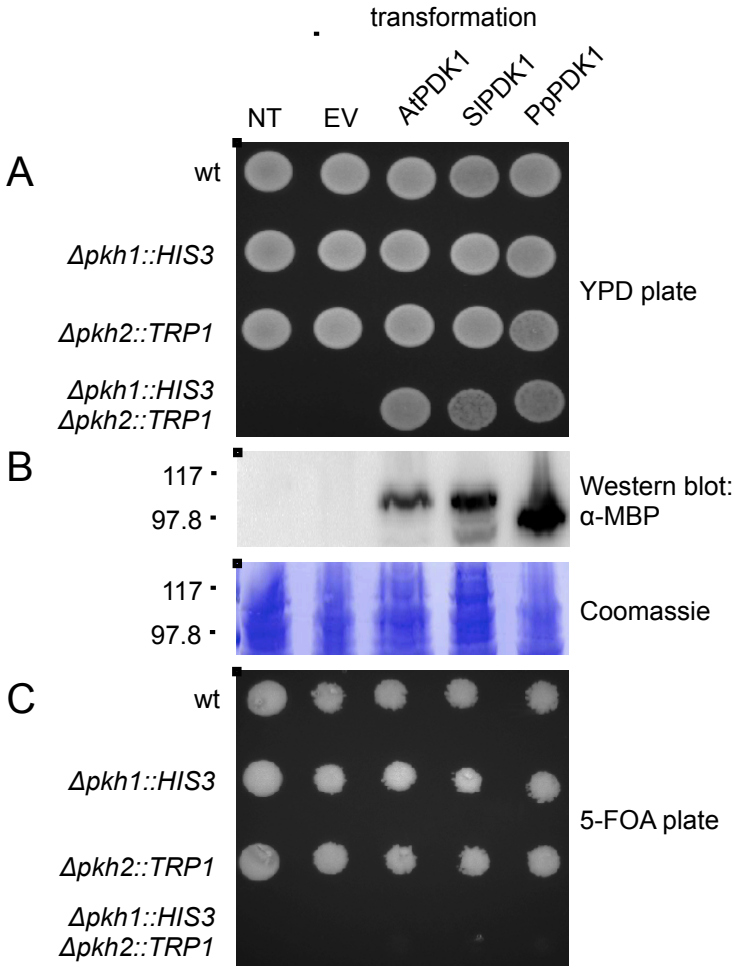

Figure 14. *PpPDK1* rescues lethality caused by deletion of both *S. cerevisiae PDK1* homologues, *PKH1* and *PKH2*. Yeast strain AC306 was transformed with p416GPD containing the indicated *PDK1* cDNAs; NT, untransformed AC306; EV, empty vector transformed AC306. Sporulation was induced and the four haploid cells produced by each diploid cell were grown on rich medium (YPD) and medium containing 5-FOA. Spore phenotypes are indicated on left and were determined by replica plating on medium lacking histidine, tryptophan, or uracil. A, On YPD plates *pkh1Δ::HIS3*/*pkh2Δ::TRP1* spores are only viable when *At*PDK1, *Sl*PDK1, or *Pp*PDK1 is expressed. B, ⟨-MBP western blot showing protein expression. C, On 5-FOA plates *pkh1Δ::HIS3*/*pkh2Δ::TRP1* spores are not viable due to loss the URA3-marked p416GPD plasmid.

(5-FOA), which induces loss of *URA3*-marked plasmids containing *PDK1* constructs. No viable *pkh1Δ::HIS3*/*pkh2Δ::TRP1* spores were recovered from 5-FOA plates (Fig. 14C), suggesting that like *At*PDK1 and *Sl*PDK1, *Pp*PDK1 is a homologue of Pkh1/2.

3.3b Use of Δpkh2 temperature sensitive yeast to show that P. patens has 1 PDK1

The *Pp174181* and *Pp188412* kinase genes identified in my search of *P. patens* for *PDK1*- like sequences appear to be more closely related to the AGC kinase S6K rather than PDK1 based on protein BLAST analysis (Fig. 11A). To verify that *P. patens* possesses a single functional homologue of PDK1, a second complementation experiment was performed with *PpPDK1*, *Pp174181*, and *Pp188412*. The previously characterized haploid *S. cerevisiae* strain INA106-3B lacks *PKH2* and possesses a point mutation in *PKH1* (D398G; *pkh2Δ::LEU2/ pkh1*^{D398G}) that confers a temperature sensitive phenotype; INA106-3B yeast is able to grow at the permissive temperature of 25°C but not at the restrictive temperature of 35°C (Inagaki et al., 1999). INA106-3B yeast were transformed with empty vector, *MBP-PpPDK1-6His*, *MBP- Pp174181*, or *MBP-Pp188412*. Transformed yeast cultures were grown in liquid medium lacking uracil at 25°C and spotted onto plates which were incubated at either 25°C or 35°C. Only yeast transformed with *MBP-PpPDK1-6His* were able to complement *pkh2Δ::LEU2/ pkh1*^{D398G}, as assessed by growth at 35°C (Fig. 11B). Western blotting with α- MBP confirmed expression of all proteins (Fig. 11B). Taken together, these results suggest that like rice (Matsui et al., 2010), and in contrast to *Arabidopsis* (Bögre et al., 2003; Camehl et al., 2011) and tomato (Devarenne et al., 2006), the *P. patens* genome contains a single *PDK1* gene.

3.4 *Pp*PDK1 does not bind phospholipids or sphingolipids

Because of its lack of a PH domain (Fig. 12A), the ability of *Pp*PDK1 to bind lipids was analyzed. *Pp*PDK1 did not appear to strongly bind phospholipids, though very weak binding to several phospholipids was detected using protein-lipid overlay assays (Fig. 15A). This result is in contrast to *At*PDK1 and *Sl*PDK1, which both strongly interact with a number of phospholipids including multiple phosphorylated phosphatidylinositols and phosphatidic acid (PA; Fig. 15A) (Deak et al., 1999). *Pp*PDK1 also appeared not to bind sphingolipids (Fig. 15B), contrary to human PDK1 which is activated by sphingosine (King et al., 2000), and Pkh1/2 which are activated by sphingoid bases (Friant et al., 2001). These results raise the possibility that, unlike the other plant PDK1s analyzed so far (Deak et al., 1999; Bögre et al., 2003; Anthony et al., 2004; Anthony et al., 2006; Zegzouti et al., 2006), *Pp*PDK1 activity and signaling may not be lipid-regulated. However, a more detailed study is required to confirm or deny lipid control of *Pp*PDK1.

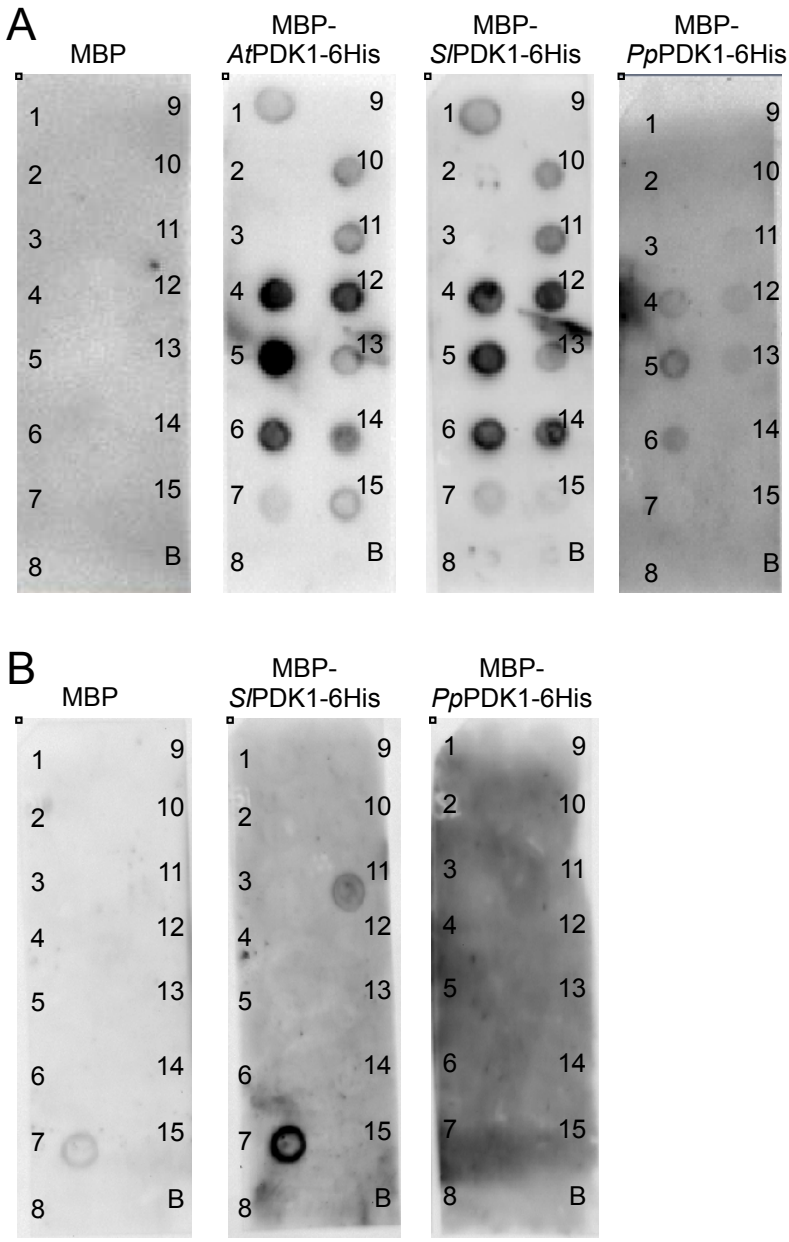

Figure 15. *Pp*PDK1 does not strongly bind phospholipids or sphingolipids. A, Five µg purified MBP, MBP-*At*PDK1-6His, MBP-*Sl*PDK1-6His, or MBP-*Pp*PDK1-6His was incubated with a membrane pre-spotted with 15 common lipids and a solvent blank, and analyzed by α-MBP western blot. Numbers indicate the lipids spotted: 1, lysophosphatidic acid; 2, lysophosphocholine; 3, PtdIns; 4, PtdIns(3)P; 5, PtdIns(4)P; 6, PtdIns(5)P; 7, phosphatidylethanolamine; 8, phosphatidylcholine; 9, sphingosine-1-phosphate; 10, PtdIns(3,4)P2; 11, PtdIns(3,5)P2; 12, PtdIns(4,5)P2; 13, PtdIns(3,4,5)P3; 14, phosphatidic acid; 15, phosphatidylserine; B, 2 methanol:1chloroform:0.8 water solvent blank. B, Five µg purified MBP, MBP-*Sl*PDK1-6His, or MBP-*Pp*PDK1-6His was incubated with a membrane pre-spotted with 15 common lipids and sphingolipids and a solvent blank, and analyzed by α-MBP western blot. Numbers indicate the lipids spotted: 1, sphingosine; 2, sphingosine-1-phosphate; 3, phytosphingosine; 4, ceramide; 5, sphingomyelin; 6, sphingosyl-phosphatidylcholine; 7, lysophosphatidic acid; 8, myriosine; 9, monosialoganglioside-GM1; 10, disialoganglioside-GD3; 11, 3-sulfogalactosylceramide; 12, psychosine; 13, cholesterol; 14, lysophosphocholine; 15, phosphatidylcholine; B, solvent blank.

3.5 Characterization of *Pp*PDK1 kinase activity

The kinase activity (auto- and *trans*-phosphorylation) of *Pp*PDK1 was tested by *in vitro* kinase assays. MBP-*Pp*PDK1-6His used either Mg^{2+} or Mn^{2+} as a divalent cation for autophosphorylation and phosphorylation of the artificial kinase substrate myelin basic protein (Fig. 16A). As expected, since Lys67 coordinates ATP, mutation of Lys67 to Gln (K67Q) abolished both *Pp*PDK1 autophosphorylation and phosphorylation of myelin basic protein (Fig. 16A). To test whether *Pp*PDK1 can activate AGC kinases, *Pp*PDK1 was incubated with either a known PDK1 AGC kinase substrate, Adi3 from tomato (Devarenne et al., 2006), or a novel putative AGC kinase isolated from *P. patens*, *Pp*2484, which was identified by searching the *P. patens* genome database at Phytozome using the Adi3 sequence (accession #AY849914). Based on ClustalW protein sequence alignment with Adi3, *Pp*2484 appears to possess characteristics of a typical AGC kinase: a kinase domain containing the invariant ATP coordinating Lys (Lys341), an activation loop within the kinase domain, and a C-terminal PIF motif for interaction with PDK1 (Fig. 17A). *Pp*2484 was further verified as a functional kinase using in vitro kinase assays to confirm the contribution of conserved residues to its activity. As expected, mutation of the conserved ATP-coordinating Lys341 to Gln (K341Q) completely abolished MBP-*Pp*2484 autophosphorylation and phosphorylation of myelin basic protein (Fig. 17B). Mutation of the conserved activation loop Ser to the phosphomimetic amino acid Asp (S577D) increased MBP-Pp2484 kinase activity compared with wild-type protein (Fig. 17B). Next, the ability of PpPDK1 to phosphorylate

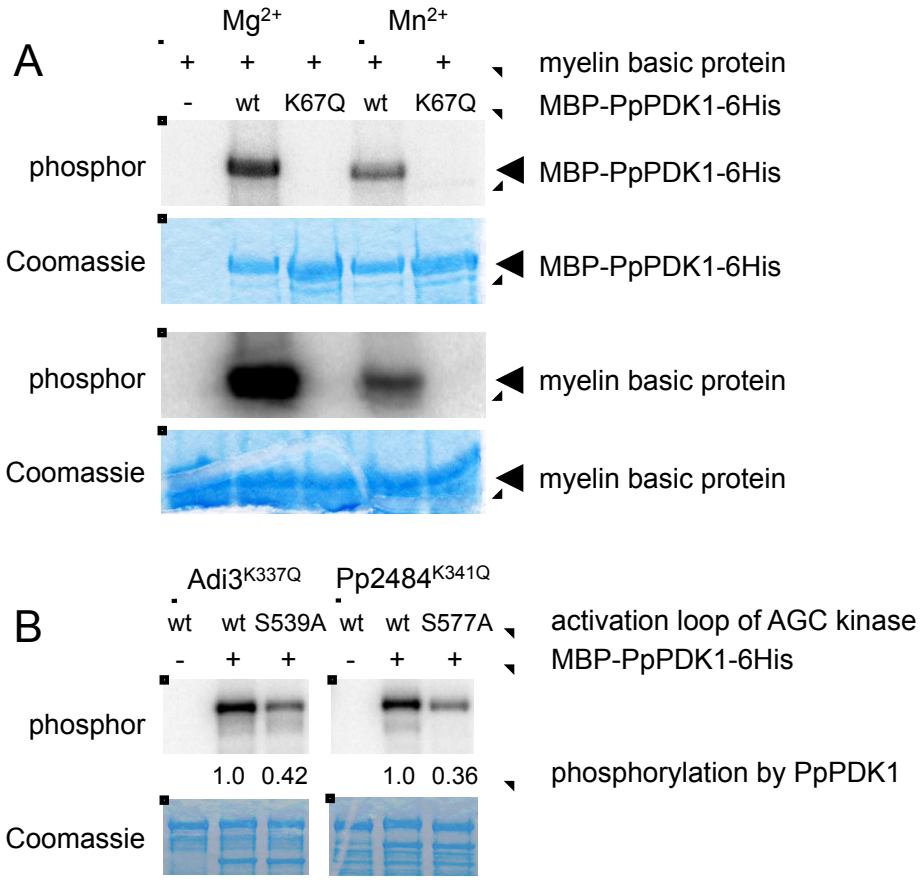

Figure 16. Characterization of *Pp*PDK1 kinase activity on myelin basic protein and AGC kinases from tomato and *P. patens*. A, Wild-type (wt) MBP-*Pp*PDK1-6His autophosphorylates and phosphorylates myelin basic protein in the presence of either Mg2+ or Mn2+, but the K67Q mutation abolishes all kinase activity. Top two panels, *Pp*PDK1 autophosphorylation; bottom two panels, *Pp*PDK1 phosphorylation of myelin basic protein. B, *Pp*PDK1 phosphorylates AGC kinases Adi3 (tomato) and *Pp*2484 (*P. patens*) at the conserved PDK1 phosphorylation site in the activation loop of both kinases. Mutation of the conserved activation loop serine to alanine (S539A in Adi3 and S577A in *Pp*2484) results in reduced phosphorylation of Adi3 and *Pp*2484 by *Pp*PDK1.

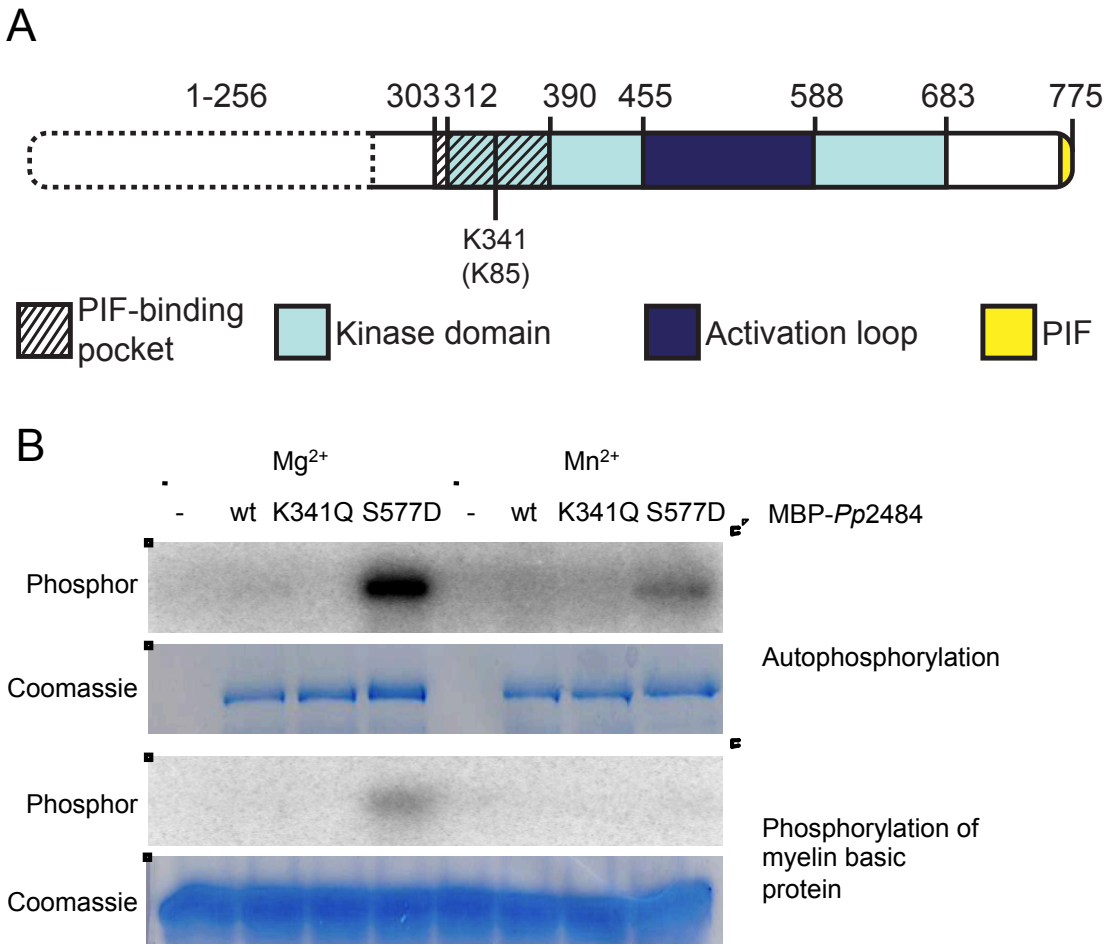

Figure 17. Characterization of kinase activity for the *P. patens* AGC kinase *Pp*2484. A, Diagram showing the protein features of *Pp*2484. B, *Pp*2484 kinase activity is consistent with an AGC kinase that is activated by PDK1 phosphorylation in the conserved activation loop serine. Wild-type (wt), kinase-inactive (K341Q), and kinase-active (S577D) MBP-*Pp*2484 were incubated with myelin basic protein in an *in vitro* kinase assay in the presence of either Mg2+ or Mn2+.

these AGC kinases was tested. MBP- *Pp*PDK1-6His phosphorylated kinase-inactive MBP-Adi3^{K337Q} and MBP-*Pp*2484^{K341Q} (Fig. 16B). As has been seen for *Sl*PDK1 phosphorylation of Adi3 (Devarenne et al., 2006), mutation of the conserved PDK1 phosphorylation site in the activation loop of MBP-Adi3^{K337Q} and MBP- Pp2484^{K341Q} (Ser539 in Adi3 and Ser577 in *Pp*2484) to Ala decreased total phosphorylation of both proteins by approximately 60 percent (Fig. 16B). These results provide evidence that, like other PDK1s, *Pp*PDK1 is able to phosphorylate AGC kinases *in vitro*, including a *P. patens* AGC kinase that is a potential substrate *in vivo*. Taken with the yeast complementation results, it seems likely that *Pp*PDK1 functions as the only PDK1 used by *P. patens* to regulate the activity of its AGC kinases.

3.6 PIF-binding pocket mutations that affect *Pp*PDK1 activity

3.6a Identification of PIF-binding pocket residues

Interaction between many AGC kinase substrates and PDK1 is mediated by hydrophobic interactions between the PIF motif at the C-terminus of the substrate and the PIF-binding pocket of PDK1 (Bögre et al., 2003). Mutating any of several important residues in the PIF-binding pocket decreases PDK1 interaction with substrates (Frödin et al., 2002). In human PDK1, the PIF-binding pocket residues that interact with the PIF motif of PKA include Lys115, Ile119, Gln150, or Leu155, and each residue is required for efficient PDK1-PIF interaction (Biondi et al., 2000). These residues correspond to Lys341, Ala345, Glu376, and Leu383 of Adi3, which also has a PIF-binding pocket that interacts with its own PIF motif, and mutation of Lys341, Gln376, or Leu383 reduces Adi3 autophosphorylation (Devarenne et al., 2006). Based on ClustalW protein sequence

alignment with human PDK1, the corresponding PIF-binding pocket residues of *Pp*PDK1 are Lys71, Ile75, Gln106, and Leu111 (Fig. 7A; Fig. 18). The DNA sequences encoding each of these residues were individually mutated to test the effects on *Pp*PDK1 phosphorylation of *Pp*2484 (Fig. 18) and Adi3 (Fig. 19), and also interaction with *Pp*2484.

3.6b Autophosphorylation of PIF-binding pocket mutants

First, the autophosphorylation activity of *Pp*PDK1 PIF-binding pocket mutants was tested by in vitro kinase assays. As has been seen in Fig. 16A, autophosphorylation was completely abolished in the kinase-inactive MBP-*Pp*PDK1^{K67Q}-6His (Fig. 18A). The K71A, K71Q, and L111A mutations all drastically reduced MBP-*Pp*PDK1-6His autophosphorylation compared to wild-type *Pp*PDK1 (Fig. 18A). In contrast, the I75A and Q106A mutations produced similar autophosphorylation levels compared to wild-type *Pp*PDK1 (Fig. 18A). These results are somewhat unexpected because PDK1 proteins lack a PIF motif, and autophosphorylation is not known to require PIF-binding pocket residues. It is possible that in *Pp*PDK1, Lys71 and Leu111 are required for the general maintenance of *Pp*PDK1 in a kinase-active state, possibly by ensuring proper conformation of the small lobe of the kinase domain where the PIF-binding pocket is found.

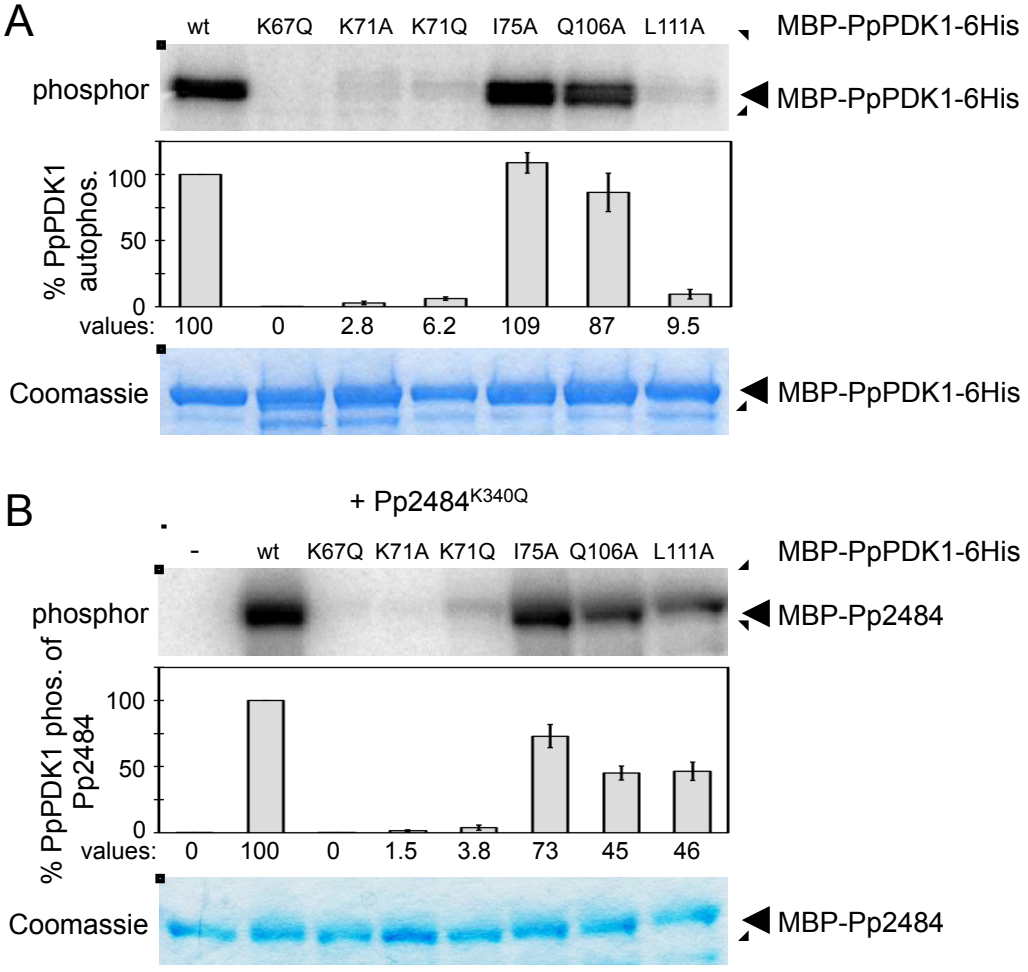

Figure 18. Functional analysis of conserved *Pp*PDK1 PIF-binding pocket residues. A, Mutation of *Pp*PDK1 PIF-binding pocket residues reduces autophosphorylation. Values are reported as the percentage of wild-type (wt) *Pp*PDK1 autophosphorylation and are the mean of three independent experiments. Error bars indicate standard error. B, Mutation of *Pp*PDK1 PIF- binding pocket residues reduces phosphorylation of *Pp*2484. Values are reported as the percentage of wt *Pp*PDK1 phosphorylation of *Pp*2484 and are the mean of four independent experiments. Error bars indicate standard error. C, Mutation of *Pp*PDK1 PIF-binding pocket residues reduces, but does not abolish, interaction with *Pp*2484. The left panel shows protein inputs analyzed by SDS-PAGE and staining with coomassie, and the right panel shows Ni+2 resin pulldowns of MBP-*Pp*PDK1-6His analyzed by α-MBP western blot. D, All *Pp*PDK1 PIF- binding pocket mutants that possess kinase activity are able to fully complement a temperature sensitive allele of *S. cerevisiae PKH1*. Haploid strain *pkh2Δ/pkh1D398G* temperature-sensitive yeast was transformed with p416GPD containing the indicated *PpPDK1* constructs. Transformed yeast were grown in liquid medium lacking uracil, spotted on plates lacking uracil, and grown at the indicated temperatures and times. Total protein was extracted from cultures grown in liquid medium at 25°C and analyzed by α-MBP western blot to verify expression of each *Pp*PDK1.

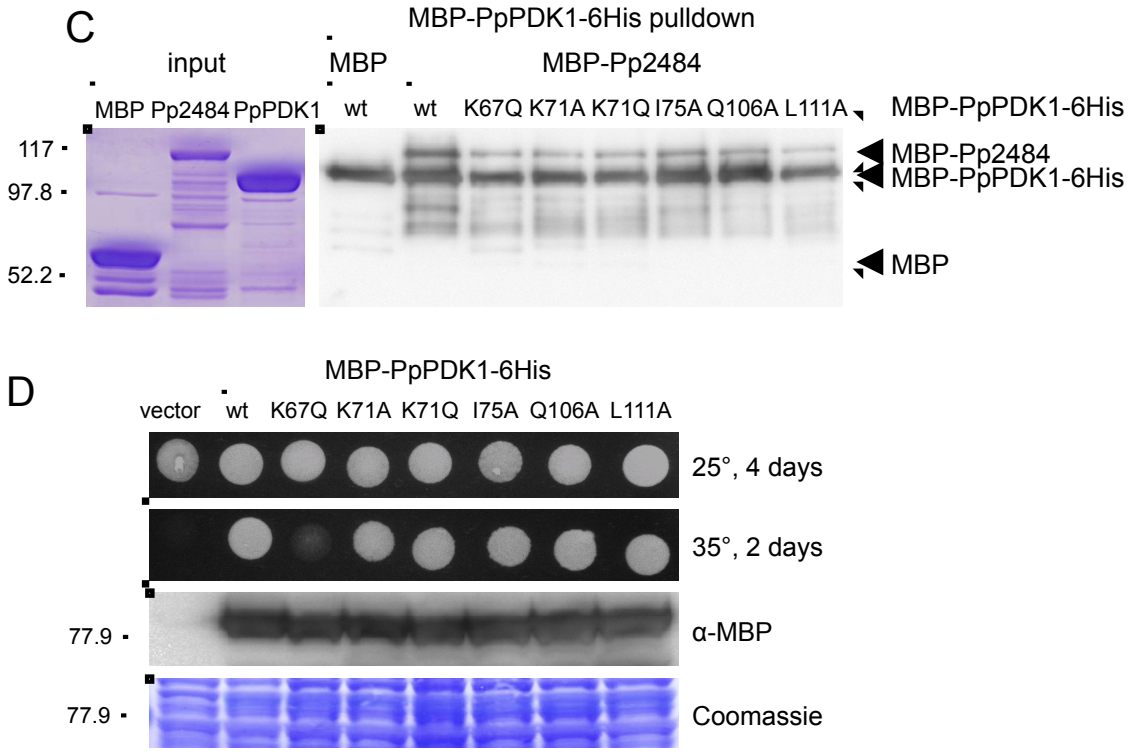

Figure 18, continued.

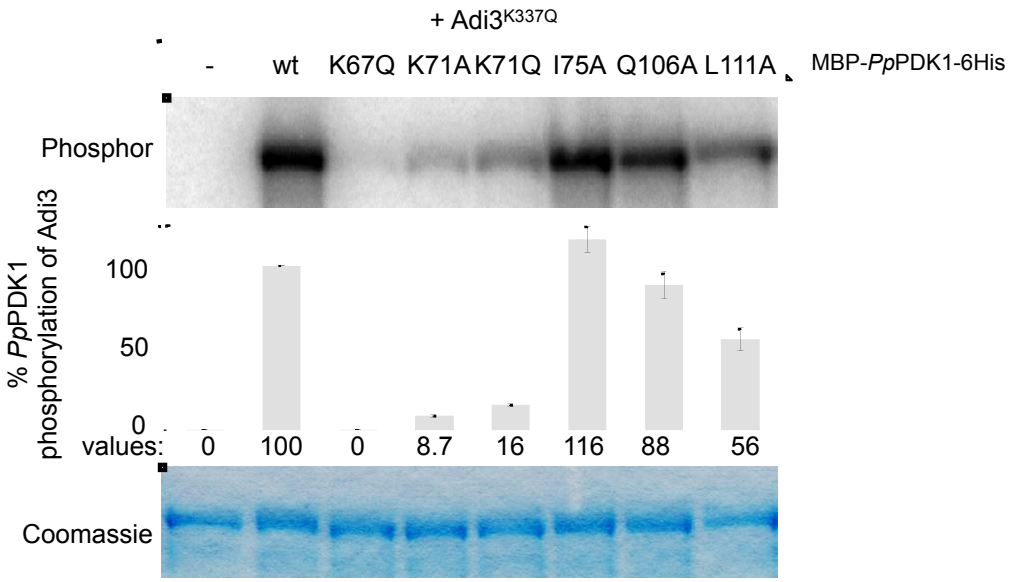

Figure 19. Phosphorylation of Adi3 by *Pp*PDK1 PIF-binding pocket mutants. Mutation of *Pp*PDK1 PIF-binding pocket residues reduces phosphorylation of Adi3. Values are reported as the percentage of wt *Pp*PDK1 phosphorylation of Adi3 and are the mean of three independent experiments. Error bars indicate standard error.

3.6c Phosphorylation of Pp2484 and Adi3

Next, the *Pp*PDK1 PIF-binding pocket mutants were incubated with kinase-inactive *Pp*2484^{K341Q} in *in vitro* kinase assays to test the activity of the *Pp*PDK1 mutants on a potential substrate. MBP-*Pp*PDK1^{K67Q}-6His displayed no detectable activity on MBP-*Pp*2484^{K341Q} (Fig. 18B). The activity of the K71A and K71Q MBP-*Pp*PDK1-6His mutants toward MBP-Pp2484^{K341Q} was drastically reduced compared to wild-type *Pp*PDK1 (Fig. 18B). The activity of MBP-*Pp*PDK1^{L111A}-6His on MBP-*Pp*2484^{K341Q} was also reduced compared to wild-type *Pp*PDK1 (Fig. 18B), but was not as dramatic as the decrease in autophosphorylation shown in Figure 18A. Interestingly, though the autophosphorylation of MBP-*Pp*PDK1^{I75A}-6His and MBP- *Pp*PDK1^{Q106A}-6His was comparable to wild-type *Pp*PDK1 (Fig. 18A), these mutants displayed reduced activity on MBP-*Pp*2484^{K341Q} compared to wild-type *Pp*PDK1 (Fig. 18B). In kinase assays containing the *Pp*PDK1 PIF-binding pocket mutants and kinase-inactive Adi3^{K337Q}, similar results were obtained for the K67Q, K71A, K71Q, and L111A mutants (Fig. 19B). However, in contrast to the kinase assays with *Pp*2484, activity of MBP-*Pp*PDK1-6His^{I75A} and MBP-*Pp*PDK1^{Q106A}-6His on MBP-Adi3^{K337Q} was comparable to wild- type *Pp*PDK1 (Fig. 19B).

3.6d Interaction of PpPDK1 mutants with Pp2484

To further investigate the contributions of Lys71, I75, Gln106, and Leu111 to substrate binding, in vitro pulldown experiments were performed between *Pp*PDK1 and *Pp*2484. Wild-type and PIF-binding pocket mutants of MBP-*Pp*PDK1-6His were pre-incubated with Ni^{2+} resin, then either α-MBP or MBP-*Pp*2484 was added, and the

interactions assessed by western blotting with α-MBP. No individual point mutation was able to abolish interaction between *Pp*PDK1 and *Pp*2484, though several *Pp*PDK1 mutants (K67Q, K71A, K71Q, and L111A) were able to pull down less *Pp*2484 than wild-type *Pp*PDK1 (Fig. 18C). Taken together with the kinase assays, these data may indicate that the analyzed *Pp*PDK1 PIF-binding pocket residues are required for proper conformation of an active kinase as well as participation in substrate binding.

3.6e Yeast complementation with PpPDK1 mutants

Finally, the ability of *Pp*PDK1 PIF-binding pocket mutants to complement Pkh1/2 was tested with the same INA106-3B yeast used to test *Pp*174181 and *Pp*188412 complementation (Fig. 11B). INA106-3B yeast were transformed with either empty vector, wild-type *MBP-PpPDK1-6His*, or a *MBP-PpPDK1-6His* PIF-binding pocket mutant and analyzed as in Fig. 10B. Unexpectedly, only yeast transformed with *MBP-PpPDK1*^{K67Q}*-6His*, which completely lacks kinase activity (Fig. 18, A and B), was unable to fully complement *PKH1/2*, as assessed by growth at 35°C (Fig. 18D). Yeast transformed with *MBP- PpPDK1*^{K71A}*-6His*, which is capable of minimal autophosphorylation (Fig. 18A) and *trans*- phosphorylation (Fig. 18B; Fig. 19), displayed comparable growth at 35°C to yeast transformed with wild- type *MBP-PpPDK1-6His* (Fig. 18D). Western blotting with α-MBP confirmed expression of all MBP-PpPDK1-6His proteins (Fig. 18D). These results suggest that, while kinase-active *Pp*PDK1 is required to perform tasks related to cell survival and growth, very low levels of activity are sufficient to enable *Pp*PDK1 to fulfill these vital functions.

3.7 Analysis of *pdk1* knockout and *PpPDK1-6His* and *PpPDK1^{K71A}-6His* transformed moss

In every organism studied so far except *A. thaliana* (Camehl et al., 2011), deletion of *PDK1* is lethal (Casamayor et al., 1999; Niederberger and Schweingruber, 1999; Rintelen et al., 2001; Lawlor et al., 2002; Devarenne et al., 2006). To test whether deletion of *PpPDK1* is lethal, I attempted to knockout *PpPDK1 via* homologous recombination using a construct containing the hygromycin resistance marker between 5' and 3' *PpPDK1* targeting fragments (Fig. 20A). At the same time I took an alternate approach in case the *pdk1* knockout line was lethal. Because *Pp*PDK1^{K71A} had the largest reduction in kinase activity (Fig. 18, A and B) but still enabled yeast viability (Fig. 18D), gene targeting was used to replace the endogenous *PpPDK1* with either *PpPDK1-6His* or *PpPDK1^{K71A}-6His* (Fig. 20B). After transformation and two rounds of antibiotic selection, the surviving moss colonies were PCR genotyped using primers 1 and 2 shown in Figure 20A and B.

3.7a Verifying PpPDK1 knockout or gene replacement

Initial genotyping showed that the majority of the *pdk1* knockout transformants did not lack the endogenous *PpPDK1* gene, whereas the majority of gene replacement transformants yielded genotyping products for both the endogenous *PpPDK1* (532 nt) and *PpPDK1-6His* (2,356 nt; data not shown), suggesting that these colonies might have been unstably transformed. Further analysis identified one *pdk1* knockout line, one *PpPDK1-6His* transformant, and one *PpPDK1^{K71A}-6His* transformant. A representative genotyping PCR is shown in Figure 20C indicating the *pdk1* knockout line lacked the

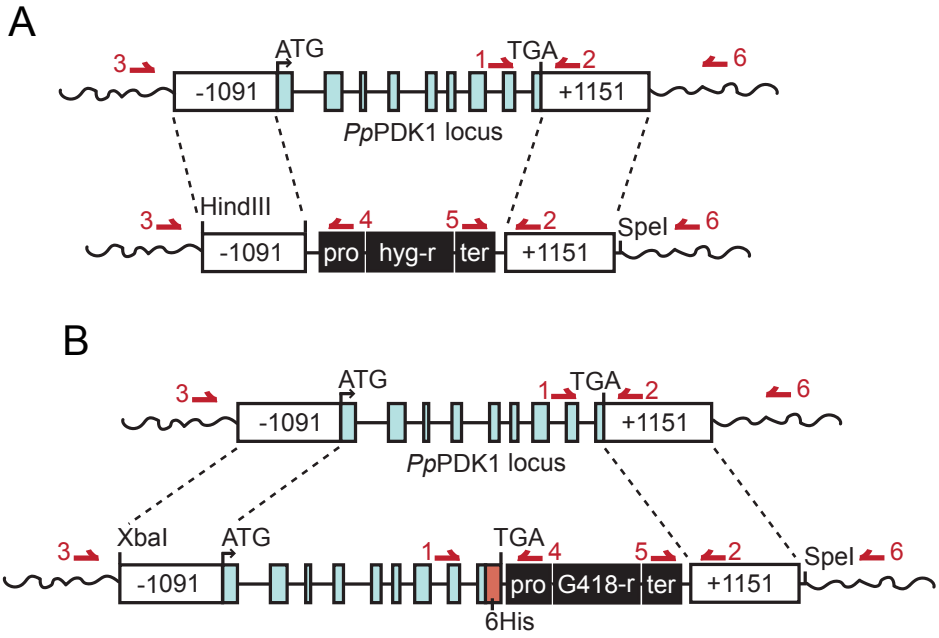

Figure 20. Production of a *pdk1* knockout line and replacement of endogenous *PpPDK1* with *PpPDK1-6His*. A and B, The constructs used to replace the endogenous *PpPDK1* locus with a hygromycin marker to create a *pdk1* knockout line (A) and to replace the endogenous *PpPDK1* locus with *PpPDK1-6His* or *PpPDK1K71A-6His* (B). The 6His tag (maroon box) is not drawn to scale for ease of visibility. Dashed lines indicate regions of homologous recombination. The locations of genotyping primers used in (C) and (D) are depicted with arrows above the diagram. C, PCR-based genotyping with primers 1 + 2 shows that the indicated *P. patens* transformants do not contain a wild-type *PpPDK1* gene. D, PCR-based genotyping of the indicated *P. patens* transformants showing proper integration of constructs into the endogenous *PpPDK1* locus. Wild-type *P. patens* genomic DNA was used as a negative control for integration of the constructs. E, RT-PCR analysis of gene expression for the indicated genes. Analysis of ubiquitin gene expression was used as an internal control.

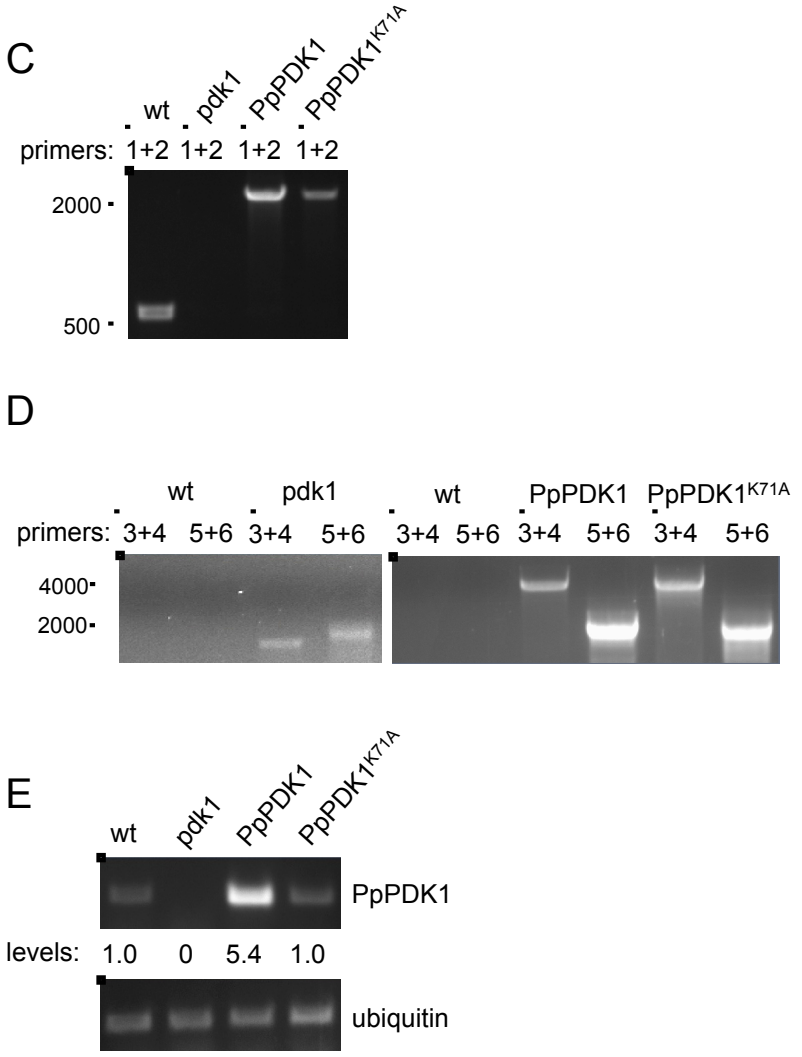

Figure 20, continued.

endogenous *PpPDK1* gene and the gene replacement lines lacked the endogenous copy of *PpPDK1* and were most likely stable transformants.

Additional genotyping was used to verify 5' and 3' integration of all constructs into the endogenous *PpPDK1* locus of the *P. patens* genome. Using the primer combinations 3 + 4 and 5 + 6 shown in Figure 19A and B, moss colonies with PCR products of the correct size were amplified from genomic DNA extracted from the *pdk1* knockout line and the gene replacement lines, but not from wild-type *P. patens* (Fig. 20D). This indicated that all transformants had integrated the exogenous DNA into the *PpPDK1* locus. These PCR products were cloned and sequenced to confirm that proper 5' and 3' integration had occurred.

Finally, Southern blot hybridization was performed to verify that *PpPDK1-6His* and *PpPDK1*^{K71A}*-6His* were present in the correct location in the *P. patens* genome. Southern blot analysis was not carried out on the *pdk1* knockout line due to a lack of large amounts of viable tissue (see below). A probe based in the 35S promoter of the G418 resistance cassette (Fig. 21A) hybridized to a predicted DNA fragment of approximately 5 kb in *Nde*I/*Sma*I/*Xba*I digested genomic DNA from *PpPDK1-6His* and *PpPDK1*^{K71A}*-6His* strains, but not from wild-type *P. patens* (Fig. 21B), confirming that the *Pp*PDK1-6His constructs had integrated into the endogenous *Pp*PDK1 locus. The *PpPDK1-6His* transformant produced four additional hybridizing DNA fragments, whereas the *PpPDK1*^{K71A}*-6His* transformant produced one additional hybridizing fragment, suggesting that both strains probably contain multiple insertions of the *PpPDK1-6His* constructs into the *PpPDK1* locus, which is a common occurrence in

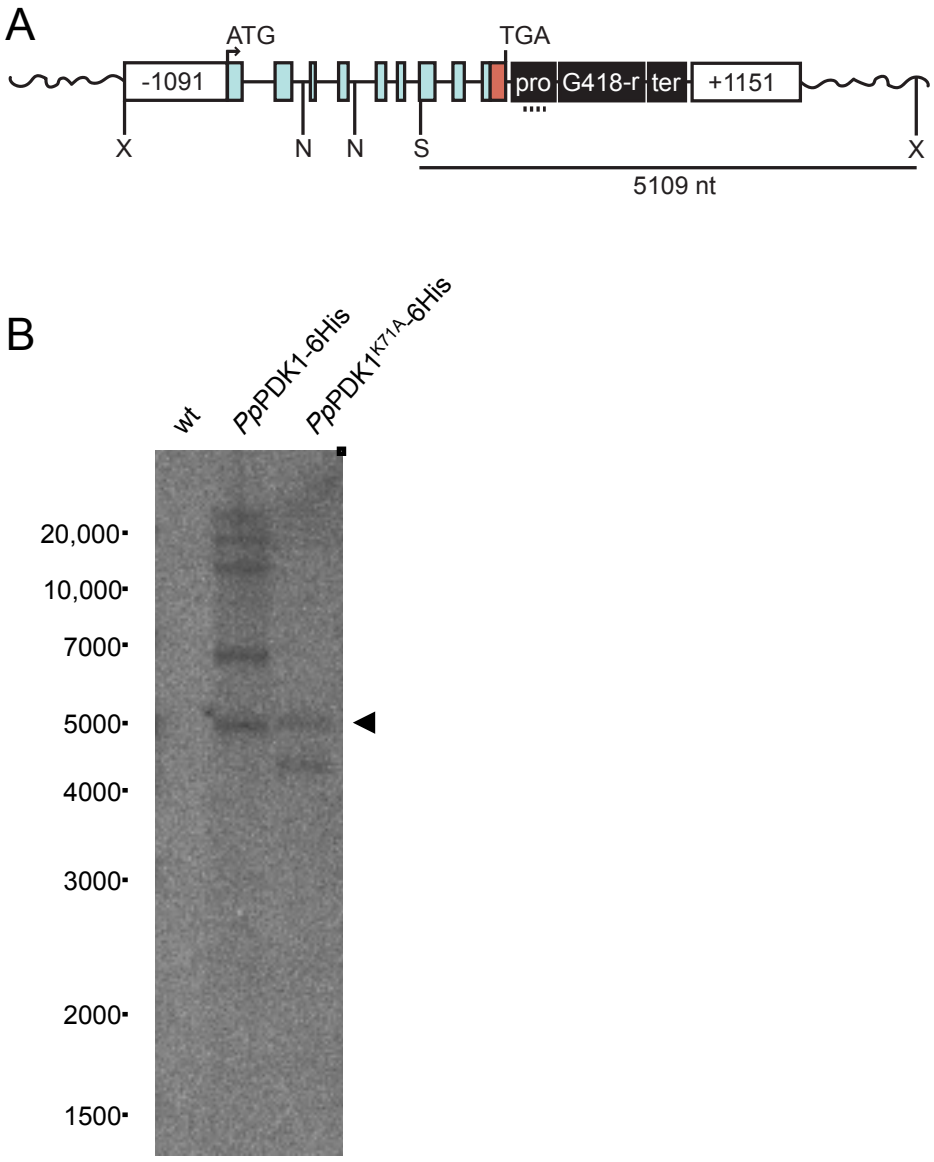

Figure 21. Southern blot analysis of *PpPDK1-6His* and *PpPDK1K71A-6His* transformed moss. A, The expected size of fragment detected by a probe based in the 35S promoter is depicted as a solid line below the figure. The location of the probe used in (B) is indicated by a dashed line. *Nde*I, *Sal*I, and *Xba*I cut sites are indicated by N, S, and X respectively. B, Southern blot analysis of genomic DNA digested with *Nde*I, *Sal*I, and *Xba*I from transformants confirms that the *PpPDK1-6His* constructs were integrated into the correct location in the genome, as indicated by the black triangle. Additional integration events, probably into the same genomic locus, also occurred in each transformant. Wild-type *P. patens* genomic DNA was used as a negative control.

transformed lines of *P. patens* (Schaefer and Zryd, 1997; Schaefer, 2002). Overall, these results indicate that the transformed moss strains lack the endogenous *PpPDK1* gene and contain at least one copy of *PpPDK1-6His* or *PpPDK1*^{K71A}*-6His* DNA that has been integrated into the *PpPDK1* locus by homologous recombination.

3.7b RT-PCR of transformed moss lines

In order to confirm loss of *PpPDK1* expression in the *pdk1* knockout line and expression of the *PpPDK1-6His* and *PpPDK1*^{K71A}*-6His* constructs, RT-PCR expression analysis was performed on all transformants as described previously using ubiquitin as a control (Harries et al., 2005). *PpPDK1* mRNA was absent from the *pdk1* knockout line and was present at similar levels in wild-type and *PpPDK1*^{K71A}*-6His* moss, whereas *PpPDK1-6His* moss contained higher levels of *PpPDK1* mRNA (Fig. 20E). These results indicate that the *pdk1* knockout line is lacking the endogenous *PpPDK1* and the gene replacement lines are expressing either *PpPDK1-6His* or *PpPDK1*^{K71A}*-6His*. Attempts to pull down *Pp*PDK1-6His and *Pp*PDK1^{K71A}-6His proteins from moss using Ni2+ resin were unsuccessful (data not shown), so future efforts to purify *Pp*PDK1 from moss may require the use of additional tags, such as a tandem affinity purification (TAP) tag, or robust overexpression of *PpPDK1*.

3.7c Phenotypes of transformed moss lines

To assess macroscopic effects from the loss of *PDK1* and the K71A mutation on *P. patens* growth and development, wild-type, *pdk1* knockout, *PpPDK1-6His*, and *PpPDK1*^{K71A}*-6His* strains were grown and allowed to develop protonema and leafy gametophore tissue over a 12 week time course. The *pdk1* knockout line and wild-type

moss grew similarly for the first 2 weeks (Fig. 22A). However, at later time points the *pdk1* knockout line produced fewer protonema, some of which began to turn brown and die (Fig. 22; Fig. 23). By 6 weeks, significant browning of the *pdk1* knockout protonema was seen and at 12 weeks much of the protonemal tissue was brown, though some green filaments were still visible (Fig. 22, A and B). A small amount of apparently healthy gametophore tissue formed in the center of the *pdk1* knockout colony (Fig. 22, A and B), but the majority of the tissue appeared to be dead.

For the gene replacement lines, the colony viability and sizes of both transformed strains appeared to be similar to wild-type moss for the first 4 weeks, after which expansion of the transformed moss colonies through protonemal growth was reduced compared to wild-type moss (Fig. 22A). At the same time, production of gametophore tissue in the gene replacement lines appeared to form earlier than the wild-type moss, but was not apparently different in morphology from the wild-type moss (Fig. 22, A and B). This result is in agreement with yeast complementation results (Fig. 18D) and suggests that the minimally active *Pp*PDK1^{K71A} is able to carry out essential functions related to moss growth and viability. It should be noted that the reduced colony size of the gene replacement moss lines could be due to improper RNA processing since the vector used for gene replacement lacks a terminator sequence for the introduced *Pp*PDK1 gene. However, this vector has been used previously for gene replacement without noticeable differences from wild-type (Shakirov et al., 2010; Spinner et al., 2010). Additionally, from the *pdk1* knockout line it appears that loss of *PpPDK1* is not completely lethal, but does not produce healthy moss tissue. In comparison, this would suggest that the

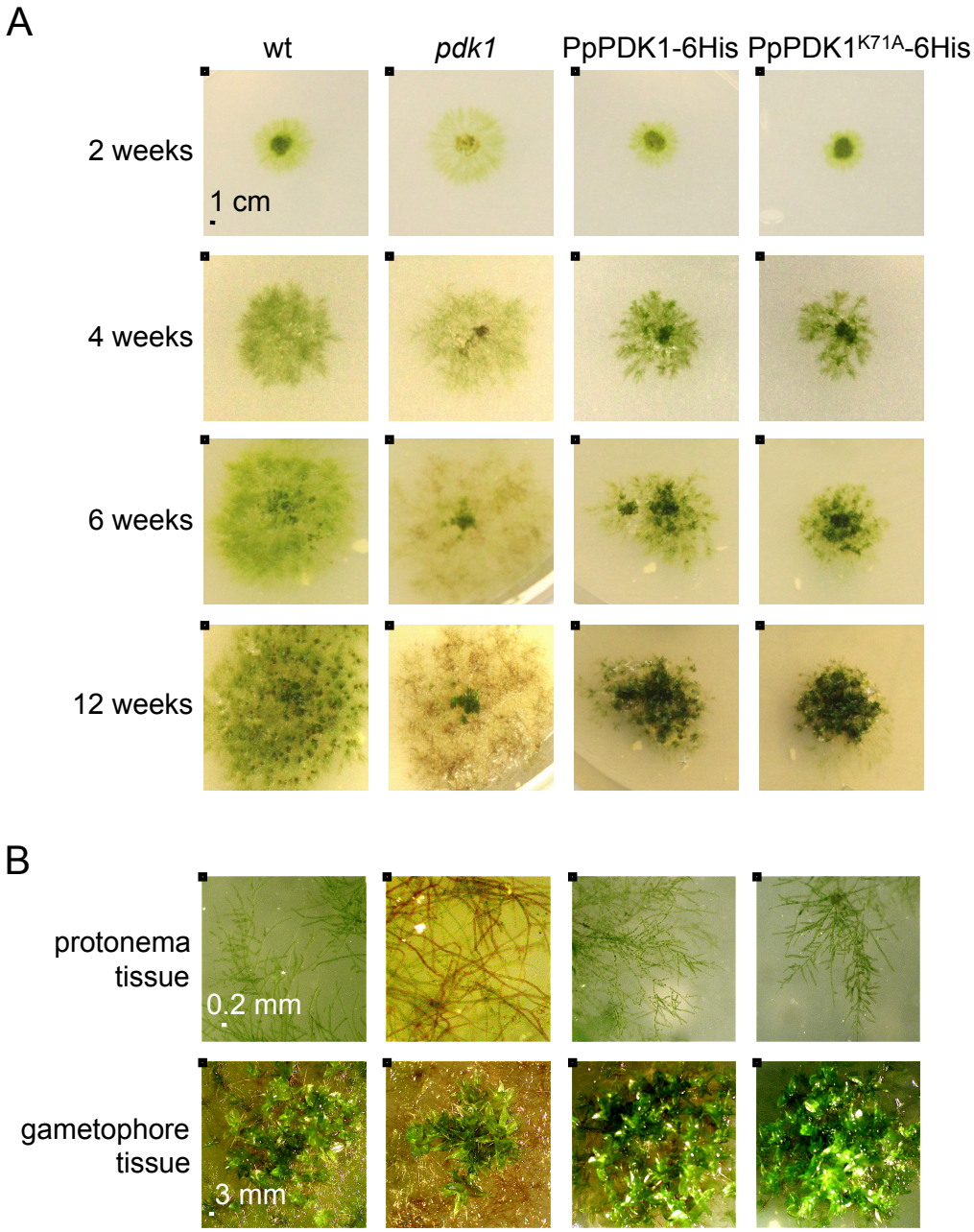

Figure 22. *pdk1* knockout, *PpPDK1-6His*, and *PpPDK1K71A-6His* moss macroscopic phenotype compared to wild-type *P. patens*. A, The indicated moss were plated and photographed over a 12 week period. B, Images of protonema and gametophore tissue for the indicated moss lines taken at the 12 week period.

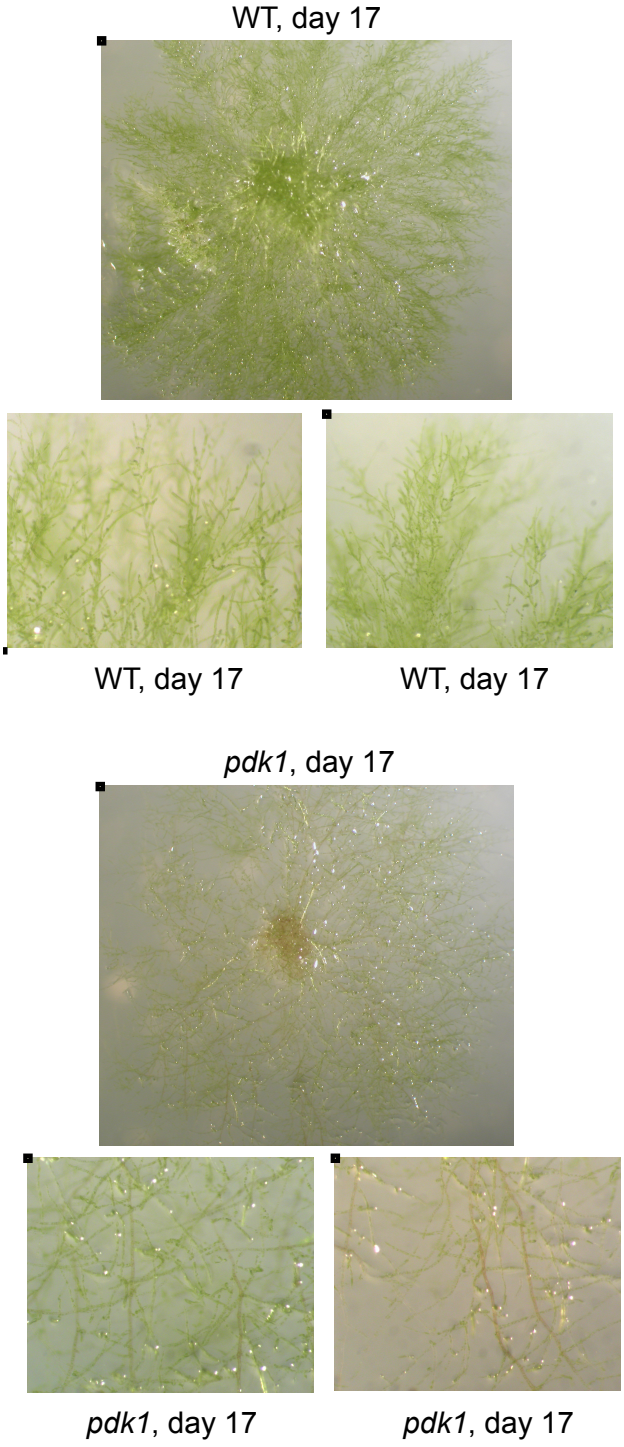

Figure 23. Protonema growth of wild-type and the *pdk1* knockout line at day 17. Moss were plated on BCD plates, grown for 17 days, and pictures taken. Pictures of the whole moss colony and close-ups of protonema from two different regions of reach moss colony are shown.

PpPDK1-6His and *PpPDK1*^{K71A}*-6His* constructs in the gene replacement lines are functional since this moss tissue was fully viable and did not show browning like the *pdk1* knockout line.

3.7d Stress treatment of transformed moss lines

As an additional test of the functionality of the *PpPDK1-6His* and *PpPDK1*^{K71A}*-6His* transformants and the impaired growth of the *pdk1* knockout line, I tested whether these moss were hypersensitive to heat (30°C) and osmotic stress (0.9 M mannitol). For heat stress, incubation at 30°C for 14 days gave surviving tissue in all moss lines except for the *pdk1* knockout line, which appeared to be completely dead (Fig. 24A). After 14 days of recovery at 25°C all moss lines except the *pdk1* knockout line were able to recover and grow healthy, viable tissue that resembled the unstressed control (Fig 24A). Similarly, the *pdk1* knockout line was most severely affected by 30 days of growth on 0.9 M mannitol and 14 days of recovery without mannitol restored growth of all lines except the *pdk1* knockout line (Fig. 24B). These data suggest that the *PpPDK1-6His* and *PpPDK1*^{K71A}*-6His* constructs in the gene replacement lines are able to function comparably to the wild-type *PpPDK1*, but that the *pdk1* knockout line is compromised in both normal growth and in response to heat and osmotic stresses.

3.8 Discussion

Regulation of many basic processes in eukaryotic cells occurs through phosphorylation of several members of the AGC kinase subfamily by PDK1 (Bögre et al., 2003; Bayascas, 2010). Thus, PDK1 function appears to be highly conserved among many different organisms suggesting *PDK1* is an evolutionarily ancient gene. While

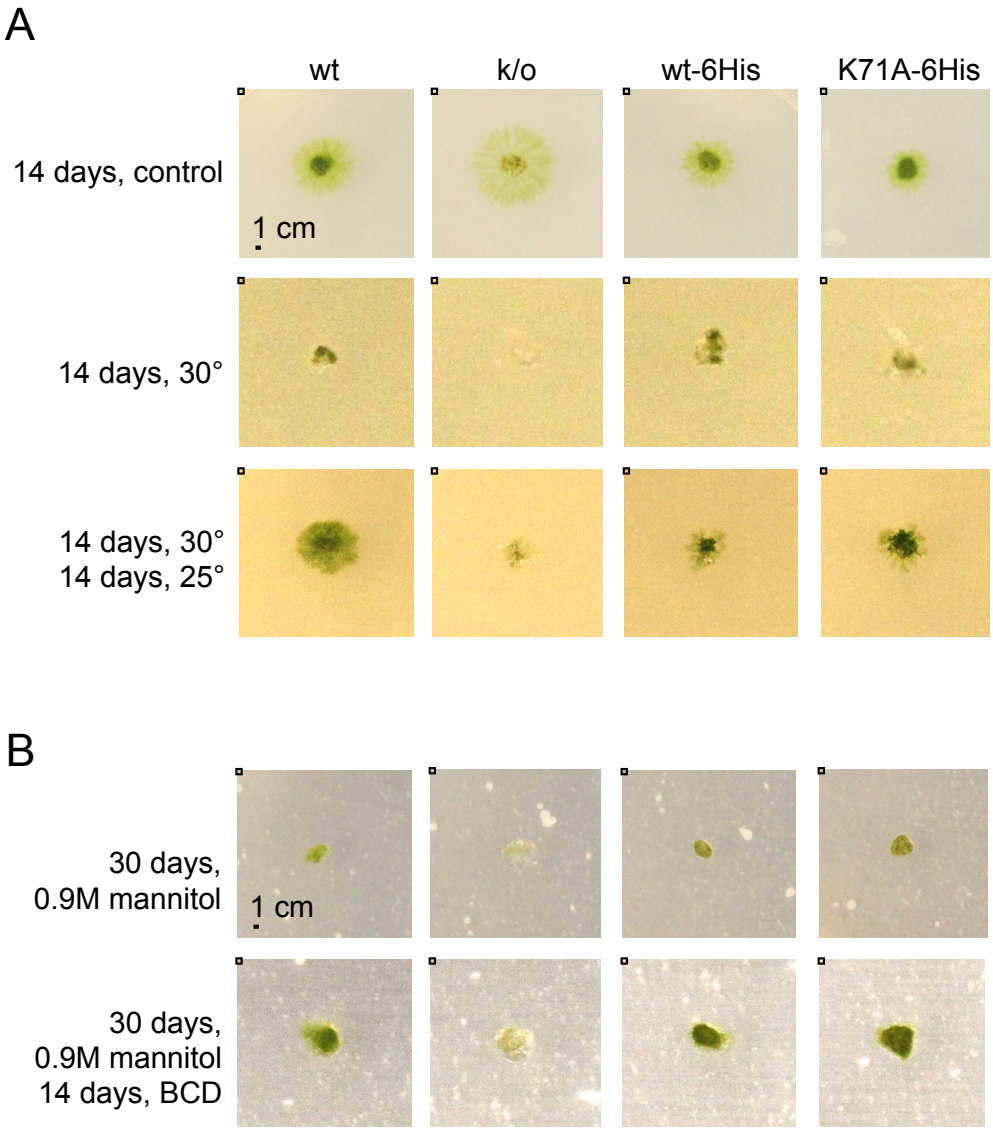

Figure 24. Stress treatment of moss lines. A, Wild-type, *pdk1* knockout, and gene replacement lines were plated on BCD plates, incubated at 30℃ for 14 days, and then moved to 25℃ for 14 days. B, Wild-type, KO, and gene replacement lines were plated on BCD plates containing 0.9M mannitol, grown for 30 days, moved to BCD plates without mannitol, and grown for 14 days.

several *PDK1* genes have been reported from higher plants (Deak et al., 1999; Devarenne et al., 2006; Matsui et al., 2010), PDK1 from organisms representative of more ancient plant systems, such as lower, non-vascular plants, has not been reported. Here I identified and characterized such a *PDK1* gene from the moss *P. patens*.

3.8a Is PpPDK1 a true PDK1?

From these studies it may be questioned whether or not *Pp*PDK1 is truly a PDK1 since it lacks a PH domain. However, in the 14 years since the human PDK1 was identified, the definition of what constitutes a PDK1 protein has emerged. Containing a PH domain does not appear to be a requirement to catalog a protein as a PDK1 since a PH domain it is not required for function of yeast Pkh1/2 (Casamayor et al., 1999). These proteins were identified as PDK1s by similarity of the kinase domains to known PDK1s, the ability of Pkh1/2 to phosphorylate known AGC kinase substrates, and by the ability of human PDK1 to rescue yeast lacking *Pkh1/2* (Casamayor et al., 1999). Thus, the conserved characteristics that define a PDK1 protein include the presence of a PIF-binding pocket and the ability to phosphorylate AGC kinase substrates at the conserved activation loop phosphorylation site (Bögre et al., 2003). Some PDK1 proteins have also been shown to phosphorylate the activation loop site on AGC kinases from a different species. For example, *At*PDK1 can phosphorylate and activate endogenous AGC kinases (Anthony et al., 2004; Anthony et al., 2006; Zegzouti et al., 2006; Zegzouti et al., 2006) as well as the mammalian AGC kinase PKB (Deak et al., 1999). PDK1 proteins from other species have also been identified by the ability of the associated gene to complement the lethality of the yeast *Pkh1/2* knockout (Deak et al., 1999).

The *Pp*PDK1 identified here meets all of these definitions of PDK1s and thus should be considered a true PDK1. *Pp*PDK1 contains a PIF-binding pocket that mediates interaction with substrates (Fig. 18C), phosphorylates *P. patens* and tomato AGC kinases at the conserved activation loop site (Fig. 16B), and can complement the yeast *Pkh1/2* knockout (Fig. 14; Fig. 18D). These complementation assays indicate that *Pp*PDK1 can regulate some of the known PDK1 functions in a heterologous system and suggests that *Pp*PDK1 may carry out the same functions within *P. patens*. These studies would also suggest that the mechanism for activation of substrates by *Pp*PDK1 is conserved with what has been discovered for other PDK1s.

3.8b Discussion of transformed moss phenotypes

Unlike other organisms, deletion of *PpPDK1* was not lethal even though much of the *pdk1* knockout moss colony appeared dead after 12 weeks of growth. Subsequent replating can recover growth of green protonema, but the same browning, non-viable phenotype appears as the moss ages. This would suggest that the *pdk1* knockout line has severe developmental abnormalities. The only other report of a non-lethal *PDK1* knockout is for *Arabidopsis* (Camehl et al., 2011). However, plants lacking both *AtPDK1-1* and *AtPDK1-2* are stunted and less fertile than wild type (Camehl et al., 2011) suggesting that a loss of *PDK1* in plants leads to growth retardation. The only other plant that was analyzed for the loss of *PDK1* was tomato, where loss of *PDK1* by virus induced gene silencing was lethal (Devarenne et al., 2006). Confirmation of lethality in tomato would require the production of a *PDK1* knockout, which is currently not possible in tomato. These contradictory results regarding the effects of *PDK1* loss in

photosynthetic organisms suggests that divergent roles for PDK1 may have evolved within plants. Further analysis on PDK1 from additional plants will be required to clarify this situation. It is also interesting to note that the minimally kinase active *Pp*PDK1^{K71A} protein could confer viability and does not produce the browning, non-viable phenotype seen with the *pdk1* knockout line (Fig. 22). The *Pp*PDK1^{K71A} protein was also capable of supporting viability in yeast (Fig. 18D).

There are several possible explanations for why *Pp*PDK1 proteins with drastically reduced kinase activity are still able to confer cell viability. First, the *Pp*PDK1 mutations did not completely eliminate interaction with a *P. patens* AGC kinase (Fig. 18C) and thus, any phosphorylation taking place could be sufficient for cell viability. Second, some functions of PDK1 may be independent of interaction with AGC kinases. For example, several non-AGC kinase proteins have been identified in mammals that are required for PDK1 function (Nakamura et al., 2008; Sephton et al., 2009). Finally, interaction of PDK1 with some substrates is independent of PIF binding (Collins et al., 2003). For example, in mammalian cells PKB, which also has a PH domain for lipid binding, appears to associate with PDK1 through the lipid interaction of their PH domains (Collins et al., 2003). This would suggest lipid binding is sufficient to activate some PDK1 substrates. However, *Pp*PDK1 does not have a PH domain (Fig. 12B) and does not strongly bind lipids (Fig. 15). Thus, it will be of interest to determine if novel mechanisms exist in *P. patens* for PDK1 interaction with substrates. Additionally, more studies with the *P. patens* PDK1^{K71A} line generated here will be needed to determine if there is altered interaction with and activation of endogenous AGC kinase substrates.

CHAPTER IV

EVOLUTION OF PDK1 FROM DIVERSE EUKARYOTES*

4.1 Rationale

Given the position of *P. patens* as a representative of early land plants, I sought to gain insights into the evolution of plant PDK1 protein domain composition and functions by comparing the *Pp*PDK1 sequence to those of other plants. Because *Pp*PDK1 is much smaller than other PDK1s reported to date and lacks a lipid-binding domain, I also wondered if *Pp*PDK1 is simply a rare exception to a highly conserved PDK1 domain architecture, or whether other PDK1 sequences might be more diverse than expected.

4.2 Features of PDK1s from algae and land plants

With a number of higher and lower plant genomes currently available it is now possible to compare the protein domain features of diverse PDK1 sequences. For the initial analysis, twenty-three PDK1 sequences were obtained from 10 vascular plants (9 angiosperms, 1 lycophyte) and 7 earlier-diverging photosynthetic species (1 bryophyte, 5 green algae, 1 red alga, 1 brown alga) for comparison (Table 3).

Next, all 23 identified PDK1 protein sequences were analyzed for conserved

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```
Cmerolae            ----------------------MMSTWRRERRTPSWAVDESWFAELERGIKGGCAAGL
Olucimarinus        ---MAEDGDDPGLVSFDELASVGTMRAEDAPRGSSPAANEEDSFGS----ATSEGQASPA
Otauri              MATATATATEDGLVNFDALATMESVRERRGRDLRIEKLREEDENSERSERSAEEGSGPGT
Osativa             ------------------------------------------------------------
Sbicolor            ------------------------------------------------------------
Zmays1              ------------------------------------------------------------
Zmays2              ------------------------------------------------------------
Athaliana1          ------------------------------------------------------------
Athaliana2          ------------------------------------------------------------
Ptrichocarpa1       ------------------------------------------------------------
Ptrichocarpa2       ------------------------------------------------------------
Rcommunis           ------------------------------------------------------------
Gmax1               ------------------------------------------------------------
Gmax2               ------------------------------------------------------------
Vvinifera           ------------------------------------------------------------
Slycopersicum1      ------------------------------------------------------------
Slycopersicum2      ------------------------------------------------------------
Ppatens             ------------------------------------------------------------
Smoellendorffii     ------------------------------------------------------------
Chlorella           ------------------------------------------------------------
Creinhardtii        ------------------------------------------------------------
Vcarteri            ------------------------------------------------------------

Cmerolae            PVDTSAKPE---------------------------------------------------
Olucimarinus        PTM---MRSVLTRKPSVAALKAM-LMSERDEEGAGEGSETDE-DEGVCSLSEFAMVLNAA
Otauri              PTEAAPMRSVLTRKPSVAALKLFSSMDVNESEAESEKMDAEEGDEDVCSLSEFAMVLNVA
Osativa             ------------------------------------------------------------
Sbicolor            ------------------------------------------------------------
Zmays1              ------------------------------------------------------------
Zmays2              ------------------------------------------------------------
Athaliana1          ------------------------------------------------------------
Athaliana2          ------------------------------------------------------------
Ptrichocarpa1       ------------------------------------------------------------
Ptrichocarpa2       ------------------------------------------------------------
Rcommunis           ------------------------------------------------------------
Gmax1               ------------------------------------------------------------
Gmax2               ------------------------------------------------------------
Vvinifera           ------------------------------------------------------------
Slycopersicum1      ------------------------------------------------------------
Slycopersicum2      ------------------------------------------------------------
Ppatens             ------------------------------------------------------------
Smoellendorffii     ------------------------------------------------------------
Chlorella           ------------------------------------------------------------
Creinhardtii        ------------------------------------------------------------
Vcarteri            ------------------------------------------------------------
```

Figure 25. Alignment of 23 plant and algae PDK1 proteins. MUSCLE (http://www.ebi.ac.uk/ Tools/msa/muscle/) was used to align protein sequences. Kinase domains are highlighted in yellow, ATP coordinating lysine is highlighted in green, and the PIF-binding pocket residues mutated in *Pp*PDK1 are highlighted in blue.

```
Cmerolae            ----------------------------------PVVCFEDSRDESEKETDQMLPHQP-
Olucimarinus        AW--DDASVGALGVEVIVKQLELLRGKIAPEASLSSMLGFDLAEDLSVRANSAGMVSVG-
Otauri              AWVRDDKLAGQLNVDVIVKQLELLRGTVGTNSSLSGVLGFDVHRDLSVRADTAGFVSIG-
Osativa             -------------------------------------MAVGGDDDMERDFAARLRLAP-
Sbicolor            -------------------------------------MAVGGDDDSMERDFAARLRLAH-
Zmays1              ------------------------------------------MERDFAARLRLAH-
Zmays2              -------------------------------------MAVGGDDDSMERDFAARLRLAH-
Athaliana1          ----------------------------------MLA-------MEKEFDSKLVLQG-
Athaliana2          ----------------------------------MLT-------MDKEFDSKLTLQG-
Ptrichocarpa1       ----------------------------------MLE-------MEREFDSKLRIQS-
Ptrichocarpa2       ------------------------------------------------------------
Rcommunis           ----------------------------------MLA-------MEKDFDSKLRIQSS
Gmax1               ----------------------------------MLE-------MEKDFDSKLKIQG-
Gmax2               ----------------------------------MLE-------TEKDFDSKLKIQG-
Vvinifera           ------------------------------------------------------------
Slycopersicum1      ----------------------------------MLALVGGEGDMEQEFDAKLKIQN-
Slycopersicum2      ------------------------------------------MEQELDSKLRIEN-
Ppatens             ------------------------------------------MAMDGTSPVSP-
Smoellendorffii     ----------------------------------MAA-------GEEEC---------
Chlorella           ------------------------------------------------------------
Creinhardtii        ------------------------------------------------------------
Vcarteri            ------------------------------------------------------------


Cmerolae            ------------PVPEVSSKTSPPVADSHSWHTDGERNARRHFIALLPGVPPLELQGAS
Olucimarinus        ------------AIGNVLYRGLSRSRSVATLSSDRERFEDILDRRAVKNISKVLGGGDE
Otauri              ------------SIANVLYRGMARARSMADMSDDLDAFENLLYKRAVKSISTVLSSGND
Osativa             -------------SPASPNAAAGGGGGGIAFRAPQEQFT--------------------
Sbicolor            ---------SPSPASPAAAASSSPTAAGGIAFRAPQEQFT--------------------
Zmays1              ---------SPSPATPAAAASSSPTAAGGIAFRAPQEQFT--------------------
Zmays2              ---------SPSPATPAAAASSSPTAAGGIAFRAPQEQFT--------------------
Athaliana1          ----------------NSSNGANVSRSKSFSFKAPQENFT--------------------
Athaliana2          ---------------NSSSNGETISRSKSFAFKAPQENFT--------------------
Ptrichocarpa1       ---------GDHPSSSSNNNNGSVQRSKSFAFRAPQENFT--------------------
Ptrichocarpa2       ----------------------------MSEHSPSLKCH--------------------
Rcommunis           NNSSSSSSISSNHNNNNNNNNIGNVQRSKSFAFRAPQENFS--------------------
Gmax1               ------------NSSSSNGAGNVQRSKSFAFRAPQENYT--------------------
Gmax2               ------------NSSSSNGGGNVQRSKSFAFRAPQENYT--------------------
Vvinifera           --------------------MVALLRSKSFAFRAPQENFT--------------------
Slycopersicum1      -------------------NSANTQRSKSFAFRAPQENFT--------------------
Slycopersicum2      -------------------NLPNPQRSKSFAFRAPQENFT--------------------
Ppatens             -----------------EPNQSKPLDPKQLVMRAPQMDFT--------------------
Smoellendorffii     ----------------APSTASSCKPGSKLTFRAPQQPYT--------------------
Chlorella           ----------------------------------MQLS--------------------
Creinhardtii        ---------------MASEEGLRDREQQEGYRAPRVTLT--------------------
Vcarteri            ----------------MAESQLEDREHQEGYRAPRVALT--------------------
```

Figure 25, continued.

```
Cmerolae            ------------------------------------------------------------
Olucimarinus        DAIITSEDLKAKLDYYGVRSSMTDIISIMTQADIESTGVVKVGDLSRLLARELEQLRGLL
Otauri              DGIVTYVDLKAKLKYFGVKSSMTDIISMMSQADIEGSGVVKVSDLSRLLARELGQLRQLL
Osativa             ------------------------------------------------------------
Sbicolor            ------------------------------------------------------------
Zmays1              ------------------------------------------------------------
Zmays2              ------------------------------------------------------------
Athaliana1          ------------------------------------------------------------
Athaliana2          ------------------------------------------------------------
Ptrichocarpa1       ------------------------------------------------------------
Ptrichocarpa2       ------------------------------------------------------------
Rcommunis           ------------------------------------------------------------
Gmax1               ------------------------------------------------------------
Gmax2               ------------------------------------------------------------
Vvinifera           ------------------------------------------------------------
Slycopersicum1      ------------------------------------------------------------
Slycopersicum2      ------------------------------------------------------------
Ppatens             ------------------------------------------------------------
Smoellendorffii     ------------------------------------------------------------
Chlorella           ------------------------------------------------------------
Creinhardtii        ------------------------------------------------------------
Vcarteri            ------------------------------------------------------------


Cmerolae            ------------------------------------------------------------
Olucimarinus        DEKVDNSKSERFRVSLDIERDGAETS--NSLLKGQATFGVLQRFPTARKDRQKPGRLLWA
Otauri              NSKTEGSTNSKTRISLEFARDGNSRTVRDCLLNGQAAYGILQRFPTARPSRKKPGRLKWS
Osativa             ------------------------------------------------------------
Sbicolor            ------------------------------------------------------------
Zmays1              ------------------------------------------------------------
Zmays2              ------------------------------------------------------------
Athaliana1          ------------------------------------------------------------
Athaliana2          ------------------------------------------------------------
Ptrichocarpa1       ------------------------------------------------------------
Ptrichocarpa2       ------------------------------------------------------------
Rcommunis           ------------------------------------------------------------
Gmax1               ------------------------------------------------------------
Gmax2               ------------------------------------------------------------
Vvinifera           ------------------------------------------------------------
Slycopersicum1      ------------------------------------------------------------
Slycopersicum2      ------------------------------------------------------------
Ppatens             ------------------------------------------------------------
Smoellendorffii     ------------------------------------------------------------
Chlorella           ------------------------------------------------------------
Creinhardtii        ------------------------------------------------------------
Vcarteri            ------------------------------------------------------------
```

Figure 25, continued.

```
Cmerolae            ------------------------------------------------------------
Olucimarinus        KDDEMCA-------------------------------------------------------
Otauri              EDEDIAAVCLFDRSGTMMIQEQGELVTLPTKLDYFTDVHRSKDTRTIVELPPKEPSEEED
Osativa             ------------------------------------------------------------
Sbicolor            ------------------------------------------------------------
Zmays1              ------------------------------------------------------------
Zmays2              ------------------------------------------------------------
Athaliana1          ------------------------------------------------------------
Athaliana2          ------------------------------------------------------------
Ptrichocarpa1       ------------------------------------------------------------
Ptrichocarpa2       ------------------------------------------------------------
Rcommunis           ------------------------------------------------------------
Gmax1               ------------------------------------------------------------
Gmax2               ------------------------------------------------------------
Vvinifera           ------------------------------------------------------------
Slycopersicum1      ------------------------------------------------------------
Slycopersicum2      ------------------------------------------------------------
Ppatens             ------------------------------------------------------------
Smoellendorffii     ------------------------------------------------------------
Chlorella           ------------------------------------------------------------
Creinhardtii        ------------------------------------------------------------
Vcarteri            ------------------------------------------------------------


Cmerolae            ------------------------------------------------------------
Olucimarinus        ------------------------------------------------------------
Otauri              PPQGFARIFACCFAPKILCFTKFPSQPVVAVSVNPPSRLGRSPGSAHAFVTARRLACRLA
Osativa             ------------------------------------------------------------
Sbicolor            ------------------------------------------------------------
Zmays1              ------------------------------------------------------------
Zmays2              ------------------------------------------------------------
Athaliana1          ------------------------------------------------------------
Athaliana2          ------------------------------------------------------------
Ptrichocarpa1       ------------------------------------------------------------
Ptrichocarpa2       ------------------------------------------------------------
Rcommunis           ------------------------------------------------------------
Gmax1               ------------------------------------------------------------
Gmax2               ------------------------------------------------------------
Vvinifera           ------------------------------------------------------------
Slycopersicum1      ------------------------------------------------------------
Slycopersicum2      ------------------------------------------------------------
Ppatens             ------------------------------------------------------------
Smoellendorffii     ------------------------------------------------------------
Chlorella           ------------------------------------------------------------
Creinhardtii        ------------------------------------------------------------
Vcarteri            ------------------------------------------------------------
```

Figure 25, continued.

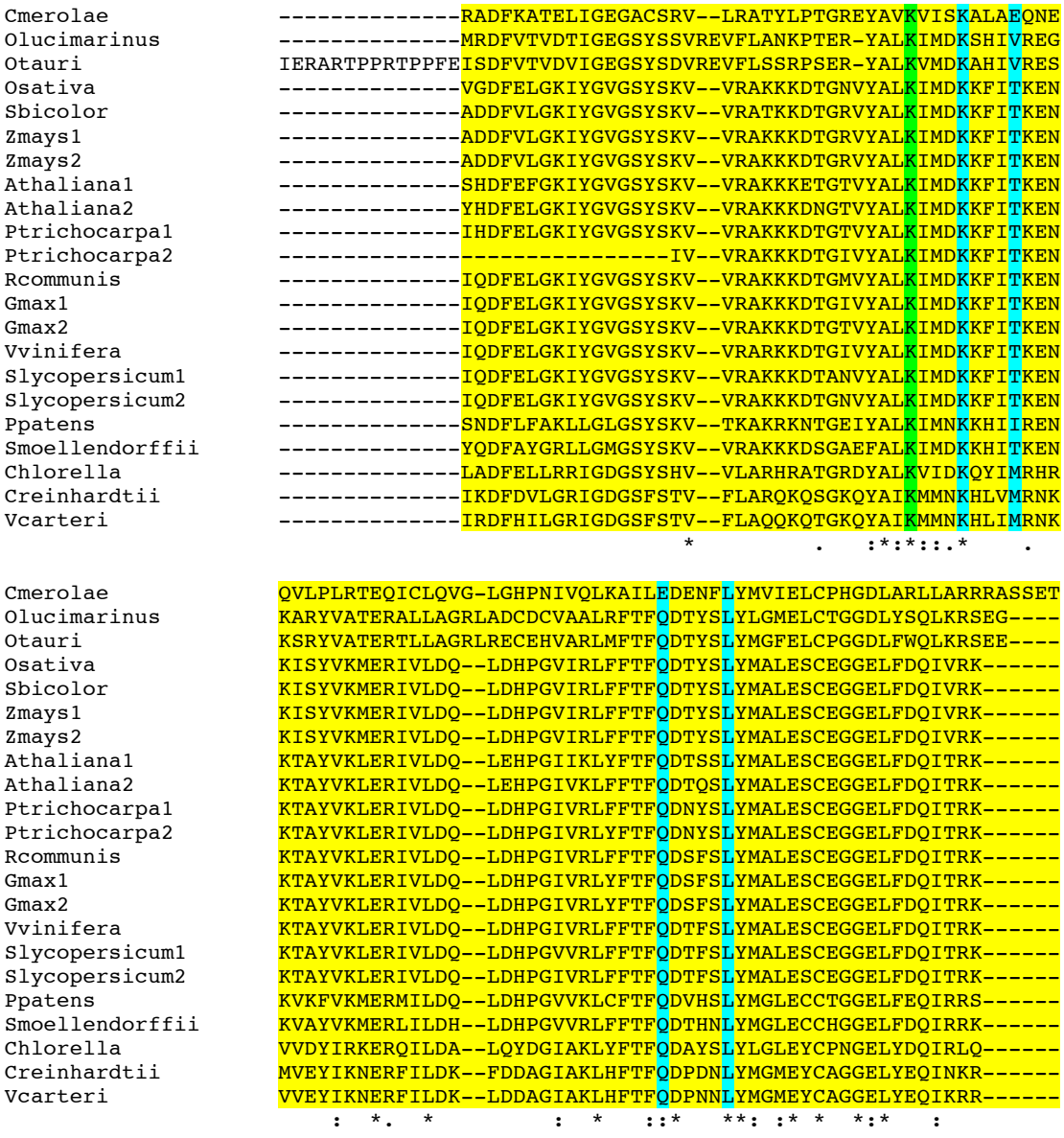


Figure 25, continued.

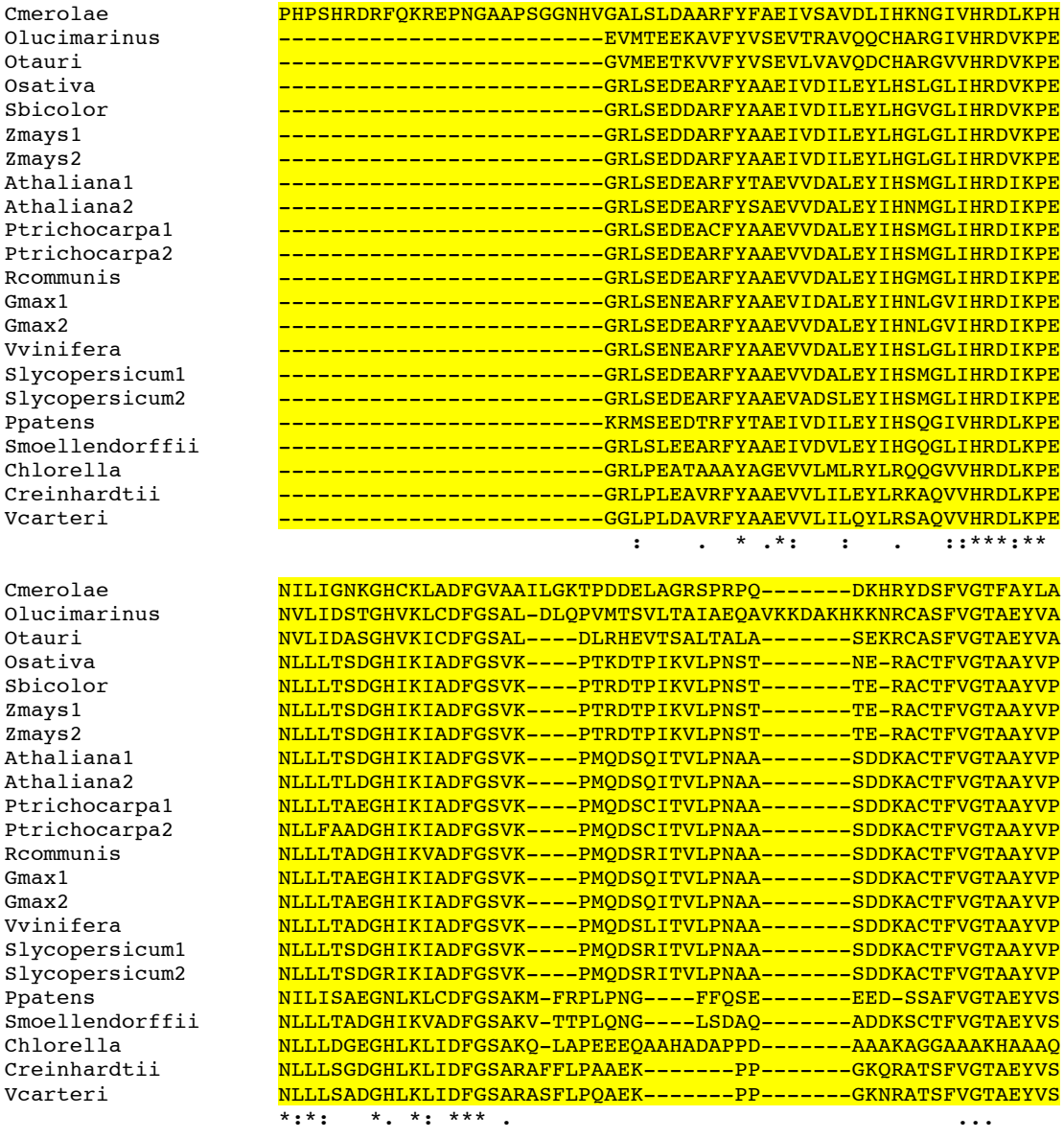


Figure 25, continued.

```
Cmerolae            P-EQLRRERPGGGFESDLWALGVVLYQMLCGGELPFRGETDYLLFQSILKDDVAF-PKSC
Olucimarinus        P-EILEG-CAETTTAVDLWSIGVMTFQLLTGRV-PFKDKTEYLTMQAVLKGKYVYPPEAN
Otauri              P-EILDG-CEETTTAVDLWSIGIMTFQLLTGRV-PFKGKT-----------DTRL-SAN-
Osativa             P-EVLNS-AP-PTFGNDLWALGCTLYQLLSGSS-PFKDASEWLIFQRIIARDLKI-PEY-
Sbicolor            P-EVLNS-AP-ATFGNDLWALGCTLYQMLSGSS-PFKDASEWLIFQRIIARDLKF-PEY-
Zmays1              P-EVLNS-AP-ATFGNDLWALGCTLYQMLSGSS-PFKDASEWLIFQRIIARDLKF-PEY-
Zmays2              P-EVLNS-AP-ATFGNDLWALGCTLYQMLSGSS-PFKDASEWLIFQRIIARDLKF-PEY-
Athaliana1          P-EVLNS-SP-ATFGNDLWALGCTLYQMLSGTS-PFKDASEWLIFQRIIARDIKF-PNH-
Athaliana2          P-EVLNS-SP-ATFGNDLWALGCTLYQMLSGTS-PFKDASEWLIFQRIIARDIKF-PNH-
Ptrichocarpa1       P-EVLNS-SP-ATFGNDLWALGCTLYQMLSGTS-PFKDASEWLIFQRIIARDIRF-PDY-
Ptrichocarpa2       P-EVLNS-SP-ATFGNDLWALGCTLYQMLSGTS-PFKDASEWLIFQRIIARDLRF-PDY-
Rcommunis           P-EVLNS-SP-ATFGNDLWALGCTLYQMLSGTS-PFKDASEWLIFQRIIARDIRF-PNY-
Gmax1               P-EVLNS-SP-ATFGNDLWALGCTLYQMLSGTS-PFKDASEWLIFQRIIARELRF-PDY-
Gmax2               P-EVLNS-SP-ATFGNDLWALGCTLYQMLSGTS-PFKDASEWLIFQRIIARDLRF-PDY-
Vvinifera           P-EVLNS-SP-ATFGNDLWALGCTLYQMLSGTS-PFKDASEWLIFQRIIARDIRF-PNY-
Slycopersicum1      P-EVLNS-SP-ATFGNDLWALGCTLYQMLSGTS-PFKDASEWLIFQRIIARDIRF-PNY-
Slycopersicum2      P-EVLNS-SP-ATFGNDLWALGCTLYQMLSGFS-PFKDTSEWLIFQRIIARDIRF-PNY-
Ppatens             P-EVLHG-KS-ASHSVDLWALGCTIYQMLEGRP-PFKAATEYLTFQKVMARELSI-PSH-
Smoellendorffii     P-EVLNG-HP-VTIGADLWALGCIIYQMLEGRP-PFKGGSEYLTFQKVLAKDLVI-PSH-
Chlorella           PGSILNN-RA-VTCAADLWALGCVVYQMLAGRP-PFKSPSEYLTFQKIVEADYEL-PEG-
Creinhardtii        P-EVLLN-AP-LSYPADLWALGCMIYQMIVGRP-PFKAASEYLTFQKITDRGLRG-PVV-
Vcarteri            P-EVLLN-QP-LSYPADLWALGCLLYQMIVGRP-PFKAASEYLTFQKITDRDFCY-PEEP
                    * . *           ***::*   :*:: *   **.  :               .

Cmerolae            LPTSSGRDLVEKLLNKDPAQRIT-------MRALKLHPFFKGI-DFRHLHRVDASKLLGP
Olucimarinus        VSSA-AKDFIDKLLVREPKKRLGF----EDETSIRSHPFFASVSDWSTLRARKAPSVL--
Otauri              ISES-AKDFIDSLLTRDPKKRLGY----ENETSIRNHPFFAAVDDWSELRSREAPRVL--
Osativa             FSDD-ARDLIDKLLDVDPSKRPGA--GPDGYVSLKKHPFFRGI-DWKNIRSTRAPKLA-M
Sbicolor            FSAE-ARDLIDKLLDVDPSKRPGA--GPDGYSSLKAHPFFRGI-DWKNLRKTRPPKLA-F
Zmays1              FSAE-ARDLVDKLLDVDPGKRPGA--GPDGYSSLKEHPFFRGI-DWKNLRKTRPPKLA-I
Zmays2              FSAE-ARDLVDKLLDVDPGKRPGA--GPDGYSSLKEHPFFRGI-DWKNLRKTRPPKLA-I
Athaliana1          FSEA-ARDLIDRLLDTEPSRRPGA--GSEGYVALKRHPFFNGV-DWKNLRSQTPPKLA-P
Athaliana2          FSEA-ARDLIDRLLDTDPSRRPGA--GSEGYDSLKRHPFFKGV-DWKNLRSQTPPKLA-P
Ptrichocarpa1       FSGE-ARDLIDHLLDIDPSRRPGA--GRGGYAELKNHPFFEGV-DWKNLRGETPPKLV-S
Ptrichocarpa2       FSEE-ARDLIDHLLDIDPSRRPGA--GRGGYAVLKNHPFFEGV-DWKNLRGETPPKLV-L
Rcommunis           FSEE-ARDIIDRLLDIDPSRRPGA--GPEGYAALKIHPFFKGI-NWKNLREETPPKLA-L
Gmax1               FSDE-ARDLIDRLLDLDPSRRPGA--GPDGYAILKSHPFFKGV-DWDNLRAQIPPKLA-P
Gmax2               FSDE-ARDLIDRLLDLDPSRRPGA--APDGYAILKRHPFFKGV-DWDNLRAQIPPKLA-P
Vvinifera           FSDE-ARDLIDRLLDTEPSRRPGA--GRDGYASLKMHPFFNGV-DWKNLRSQTPPKLA-M
Slycopersicum1      FSNE-ARDIIDQLLDVDPSRRPGA--GPDGYASLKNHPFFSGI-DWENLRLQTPPRLA-M
Slycopersicum2      FSNE-ARDLIDQLLDIDPSRRPGA--GPDGYASLKNHPFFSGV-DWDNLRLQTPPRLA-A
Ppatens             FSPE-AKDLVDSLLNLKPNERLGV----QGYDDIKNHPFFKGF-DWSRLRKMATPKLL-K
Smoellendorffii     FPSA-AKELINKLLNLEPDKRPGA--GPAGYTALKSHAFFSGI-EWLKLRQSAAPGLA-P
Chlorella           GSEA-AADLVARLLRVEPAQRIGA----ADLAELQAHPFFAGI-DWDTLRSQPAPEFM-P
Creinhardtii        YPDD-ARDLTDRLLTMEPAARIGEWRSAEDMAELRAHPFFAGV-DWAALRAGPAPPYL-P
Vcarteri            ATAA-ARDLTDRLLAMEPSARIGE-SCAEDISELKAHPFFAVI-DWETLRSGPAPSFL-P
                     .   . ::   **  .*  *           :. *.**  . ::  :.   ..
```

Figure 25, continued.

```
Cmerolae            DAFEKRDFLSFDAASSAGHAAEGFRRVTRAINS-WWPRGTHAHRRATREPQALQDAGAKE
Olucimarinus        ----------------------TATGVGSDATVSESESESDTDD-----------------
Otauri              ----------------------TATGVGSDATVTDSECDHDDGTDT---------------
Osativa             EANA------NEDEDSQDSSW-LSHMGSAPVNQHVSPVGNDGASSSSEVRSHISRLASID
Sbicolor            DANA------NEDEDGQDSNW-LAHMGTVAVNQQSNTVGNNGAPSSSEVRSHISKLSSID
Zmays1              DANA------NEDEDSQDSNW-LAHMGSVAVNQQSNTVGNNGAPSSSEVRSHISKLASID
Zmays2              DANA------NEDEDSQDSNW-LAHMGSVAVNQQSNTVGNNGAPSSSEVRSHISKLASID
Athaliana1          DPASQT--ASPERDDTHGSPWNLTHIGDSLA---TQNEGHSAPPTSSESSGSITRLASID
Athaliana2          DPASQS--ASPERD---GSPWNPTHVGDTSV---LQNDGHNGL---SESSGSITRLASID
Ptrichocarpa1       EPMVQS----GDSDHDSGSPYNPTRAGDSSL---TQNDGNAGVSSSAEATAHITRLASID
Ptrichocarpa2       EPMAQS----GDNDHDPGSPFNPTHIGDSSM---TQNDANVGVSSSAEATSHIARLASID
Rcommunis           EAMTQS----GDGELDPDATWNPTHIGDGSA---RQNDGTGGPSSTAEASGSITRLASID
Gmax1               EPGTQS----PASDDVHDSSWSPSHIGDGSAASVRQPDG----ATSSEGTGHITRLASID
Gmax2               EPGTQS----PVADDVHDSSWSPSHIGDGSAASVRQPDG----ATSSEGTGHITRLASID
Vvinifera           EAMAHS----SEGDDGQDS-WNPAHIGDGSA---RQNDGNSGATSSSEAPGSVTRLASID
Slycopersicum1      EPKAPS---THSSGDEQDPSWNPSHIGDGSV---RPNDGNGAAASVSEAGNSITRLASID
Slycopersicum2      EPKGHS---TRTSGEDHDPSWNPSHIGDGSA---RPNDGNGGTPSSSEA-NSVTRLASID
Ppatens             DPNTES----LDEE------------------------------------------------
Smoellendorffii     SPSSQGGGNGAESDDSENSDWDLAHLGGRVS-RLDVSDASSPMSSSNEPSSPSAVLASAA
Chlorella           D---------IDPGEQLVGSWRAAGIPA--------------------------------
Creinhardtii        PRVPGA--PGSDGGYEEGLDWELTSLVRDAA------------------------------
Vcarteri            PTPPGG-----AGDGEEGWDWELTSLVRDAA------------------------------


Cmerolae            LF---AAHEKWRQ---PARRVLNLASDFTGNPILASAIELVESLLQRIRRECMQHRKLPS
Olucimarinus        -----D--EEWRA-------------------------------------------------
Otauri              -----D--DEWRA-------------------------------------------------
Osativa             SF---D--SRWQDFLEPGESVVLISKLKKINKLTNKKVQLI--LTDKPQLICVDPGKMVT
Sbicolor            SF---D--SKWQEFLDPGESVVLISKLKKINKLANKKVQLI--LTDKPQLICVDPSKMVA
Zmays1              SF---D--SKWQEFLDPGESVVLISKLKKINKLANKKIQLI--LTDKPQLICVDPSKMVA
Zmays2              SF---D--SKWQEFLDPGESVVLISKLKKINKLANKKIQLI--LTDKPQLICVDPSKMVA
Athaliana1          SF---D--SRWQQFLEPGESVLMISAVKKLQKITSKKVQLI--LTNKPKLIYVDPSKLVV
Athaliana2          SF---D--SRWQQFLEPGESVLMISAVKKLQKITSKKVQLI--LTNKPRLIYVDPSKLVV
Ptrichocarpa1       SF---D--SKWQQFLDPGESVLMIAMVKKLQKLTSKKVQLI--LTNKPKLIYVDPSKLVV
Ptrichocarpa2       SF---D--SKWQQFLDPGESVVMISMVKKLQKLTSKKVQLI--LTNKPKLIYVDPSKLIV
Rcommunis           SF---D--SKWQQFLDPGESVLMISMVKKLQKLTSKKVQLI--LTNKPKLIYVDPSKLLV
Gmax1               SF---D--SKWQQFLEPGESVLMISMVKKLQKLTSKKVQLI--LTNKPKLIYVDPSKLIV
Gmax2               SF---D--SKWQQFLEPGESVLMISMVKKLQKLTSKKVQLI--LTNKPKLIYVDPSKLIV
Vvinifera           SF---D--SKWQQFLEQGESVLMISMVKKIQKLTNKKVQLI--LTNKPKLIYVDPAKLMV
Slycopersicum1      SF---D--SKWKQFLDPGESVLMISMVKKLQKLTSKKVQLI--LTNKPKLIYVDPSKLVI
Slycopersicum2      SF---D--SKWKQFLEPGESVLMISNVKKIQKLTSKKVQLI--LTNKPKLIYVDPSKLVV
Ppatens             --------EKWQAGIIDGLDAFVYDV-----------------------------------
Smoellendorffii     RLLDENVHEPWQKFLFEGETILASSRVRKFRKLSVKKRQLI--LTDRPRLFYVHPIKLVF
Chlorella           --------TYW---------------------------------------------------
Creinhardtii        -----E--S-----------------------------------------------------
Vcarteri            -------------------------------------------------------------
```

Figure 25, continued.

```
Cmerolae            EEYLFAASQLQSADPRTLTDDHAKIRFWVVLYNVMFIHVRMVHGAPVRSGLRDNRFFGEY
Olucimarinus        ------------------------------------------------------------
Otauri              ------------------------------------------------------------
Osativa             KGNIMWSDDPSELNVQVSNSSHFRI----------------------CTPKKVSSFEDA
Sbicolor            KGNIIWSDDPSELSVQVSDSSHFRI----------------------CTPKKVSTFEDA
Zmays1              KGNIIWSDDPSELSVQVSDSSHFRI----------------------CTPKKVSTFEDA
Zmays2              KGNIIWSDDPSELSVQVSDSSHFRI----------------------CTPKKVSTFEDA
Athaliana1          KGNIIWSDNSNDLNVVVTSPSHFKI----------------------CTPKKVLSFEDA
Athaliana2          KGNIIWSDNSNDLNVQVSSPSHFKI----------------------CTPKKVLSFEDA
Ptrichocarpa1       KGNIIWSDNSDDLSVQVTSPSHFKI----------------------CTPKKVRSFEDA
Ptrichocarpa2       KGNIIWSDNSDDLSVQVTSPSHFKI----------------------CTPKKVRSFEDV
Rcommunis           KGNIIWSDNSNDLSVQVSSPSHFKI----------------------FTPKKVMSFEDA
Gmax1               KGNIIWSDNPNDLSIQVASPSNFKI----------------------CTPKKVMSFEDA
Gmax2               KGNIIWSDNPNDLSIQVASPSNFKI----------------------CTPKKVMSFEDA
Vvinifera           KGNIIWSDNPNDLSIQVTSPSHFKI----------------------CTPKKVMSFEDS
Slycopersicum1      KGNIIWSDNPNDLSIQVTSPSQFKI----------------------CTPKKVMSFEDA
Slycopersicum2      KGNIIWSDNSNDLNIQVISPSQFK-------------------------PKKVMSFEDA
Ppatens             ------------------------------------------------------------
Smoellendorffii     KGEVPWS---RDIYVRVENDLKFCI----------------------CTPKRTYNLEDT
Chlorella           ------------------------------------------------------------
Creinhardtii        ------------------------------------------------------------
Vcarteri            ------------------------------------------------------------


Cmerolae            FYRVFTLDYCLDDIENGILRVPGPFFRSWERDDPRRELALKHLHPKIVDMLHRIRVSRGM
Olucimarinus        --RVNAATAALDAL---------------------------------------------
Otauri              --RVNAAAAALDAL---------------------------------------------
Osativa             KQRAWQWKKAIEDLQRCQKN---------------------------------------
Sbicolor            KQRAWQWKKAIEDLQRGQRN---------------------------------------
Zmays1              KQRAWQWKKAIEDLQRCQRN---------------------------------------
Zmays2              KQRAWQWKKAIEDLQRCQRN---------------------------------------
Athaliana1          KQRASVWKKAIETLQNR------------------------------------------
Athaliana2          KQRALQWKKAIETLQNR------------------------------------------
Ptrichocarpa1       KQRAWQWKKAIESLQNQ------------------------------------------
Ptrichocarpa2       KQRAWQWKKAIESLQNQ------------------------------------------
Rcommunis           KQRAWQWKKAIESLQNQ------------------------------------------
Gmax1               KQRAWQWKKAIEGLQNR------------------------------------------
Gmax2               KQRACQWKKAIEGLQNR------------------------------------------
Vvinifera           KQRAWQWKKAIEGLQNR------------------------------------------
Slycopersicum1      KNRAQQWKKAIEALQNR------------------------------------------
Slycopersicum2      KQRAMQWKKAIETLQNR------------------------------------------
Ppatens             ------------------------------------------------------------
Smoellendorffii     KGQARVWKESIEKLVNAK-----------------------------------------
Chlorella           ------------------------------------------------------------
Creinhardtii        ------------------------------------------------------------
Vcarteri            -----------DGL---------------------------------------------
```

Figure 25, continued.

```
Cmerolae              ADIPELDHLTETNLLGQAAFEDDGLIETDTERTTLTL
Olucimarinus          ------------------------------------
Otauri                ------------------------------------
Osativa               ------------------------------------
Sbicolor              ------------------------------------
Zmays1                ------------------------------------
Zmays2                ------------------------------------
Athaliana1            ------------------------------------
Athaliana2            ------------------------------------
Ptrichocarpa1         ------------------------------------
Ptrichocarpa2         ------------------------------------
Rcommunis             ------------------------------------
Gmax1                 ------------------------------------
Gmax2                 ------------------------------------
Vvinifera             ------------------------------------
Slycopersicum1        ------------------------------------
Slycopersicum2        ------------------------------------
Ppatens               ------------------------------------
Smoellendorffii       ------------------------------------
Chlorella             ------------------------------------
Creinhardtii          ------------------------------------
Vcarteri              ------------------------------------
```

Figure 25, continued.

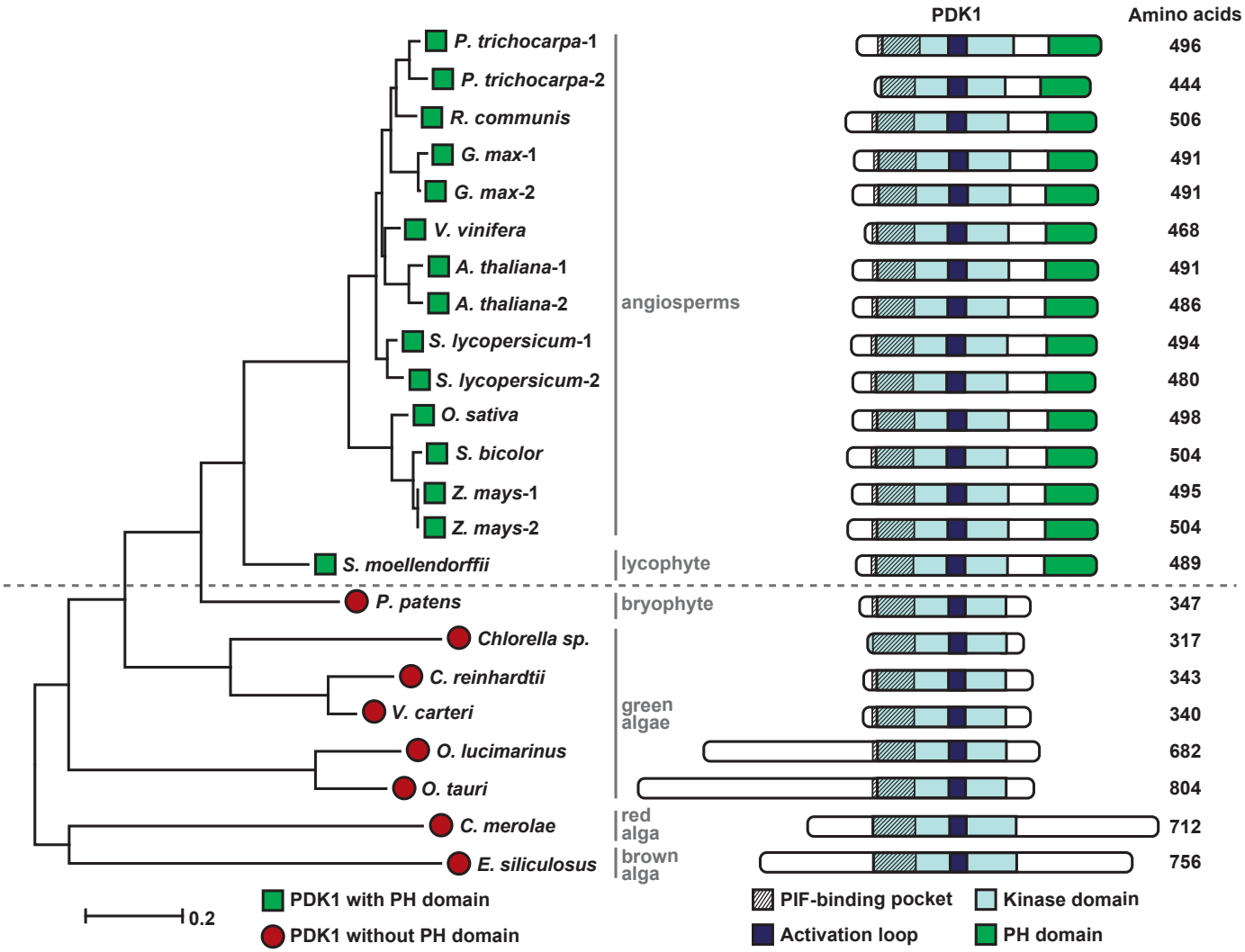


Figure 26. Phylogenetic relationship and protein domains of PDK1 proteins from plants and algae. A, Cladogram showing evolutionary relationship of plants used in B. B, Comparison of PDK1 protein sequences. On the left side, the indicated proteins were aligned and a maximum- likelihood phylogenetic tree produced using MEGA5 (Tamura et al., 2011). On the right side, the indicated domains were identified in each protein and aligned based on the activation loop domain. Horizontal dashed line indicates split between vascular (above line) and non-vascular (below line) plants.

features, including kinase domain, PIF-binding pocket, activation loop, and PH domain. Multiple sequence alignment revealed that the kinase domain of every PDK1 analyzed possesses conserved PIF-binding pocket residues (Fig. 25), but that algal and *P. patens* PDK1s differ from vascular plant PDK1s in the lack of a PH domain (Fig. 26). As might be expected, a maximum-likelihood tree constructed from all 23 plant PDK1 sequences closely resembled the known phylogenetic placement of these plants, with higher plant PDK1s much more closely related to each other than to primitive land plant or algal PDK1s (Fig. 26). This sequence analysis suggests that, while many residues in the PDK1 catalytic domain and PIF-binding pocket have been maintained in highly divergent plant taxa throughout hundreds of millions of years of evolution, the lipid-binding PH domain may only be required in higher plant PDK1s, and is not a characteristic feature of PDK1s from non-vascular land plants and algae.

4.3 Analysis of putative PDK1s from diverse eukaryotes

4.3a Selection of PDK1 sequences for analysis

Because of the initial unexpected finding that putative PDK1s from vascular and non-vascular plants seem to be distinguished by the presence of absence of a PH domain, my next goal was to identify additional trends in PDK1 features by investigating PDK1 homologues from much more distantly related eukaryotes. Putative PDK1 protein sequences from 100 different species of eukaryotes, including 35 photosynthetic and 65 non-photosynthetic organisms, were obtained from BLAST searches of NCBI GenBank and genome databases (Table 4). Some organisms possess multiple putative PDK1s, but only the top BLAST hit from each species was selected for further analysis. I did not

include an exhaustive list of putative PDK1s from each species for several reasons: 1) space considerations; 2) genome analysis tools, particularly for the most recently sequenced genomes, limit our ability to confidently assert the number of the putative PDK1s present in a given organism; 3) though abundant evidence suggests that every eukaryote is likely to have at least one PDK1 (Goldberg et al., 2006; Manning et al., 2002a; Manning et al., 2011; Judelson and Ah-Fong, 2010; Wang et al., 2003; Van Dam et al., 2011), not all organisms have more than one PDK1. Thus, I chose the simplistic strategy of analyzing only the top BLAST hit from each species, as the highest-scoring sequence is most likely to represent a true PDK1 homologue. In my opinion, the true test of a PDK1 is its ability to phosphorylate AGC kinase substrates at the conserved activation loop site. This test must be experimentally performed with each putative PDK1, so I am hesitant to attempt to differentiate high-scoring BLAST hits from each other solely based on sequence analysis. This approach is a conservative one, which makes it impossible to assess instances of possible PDK1 functional divergence in organisms with multiple putative PDK1s, a topic that should be investigated in the future.

4.3b Analysis of PDK1 PIF-binding pocket and catalytic domain sequences

All 100 PDK1 protein sequences were aligned as previously described (Dittrich and Devarenne, 2012). The PIF-binding pocket regions of all proteins in this alignment were visually inspected for the presence or absence of 9 amino acids demonstrated to participate in substrate interaction (Frödin et al., 2002). Six of these amino acids (corresponding to F82, K115, R131, F149, Q150, and L155 of human PDK1) are

conserved in almost all putative PDK1 sequences, whereas 3 amino acids (corresponding to K76, I119, and F147 of human PDK1) are not well conserved (Fig. 27). Eighty-four of the putative PDK1 sequences have a conserved amino acid at all 6 highly-conserved PIF-binding pocket positions, while 16 putative PDK1 sequences contain a non-conserved amino acid at one or more positions. In addition, 3 putative PDK1 sequences were identified that might be incomplete at the N-terminus: *Trichoplax adhaerens* PDK1 does not start with methionine, and *Monosiga brevicollis* and *Micromonas sp.* PDK1s appear to lack at least one amino acid in the N-terminal part of the PIF-binding pocket (Fig. 27).

After alignment and phylogenetic analysis, sequence similarity within the most highly conserved regions of putative PDK1s, the catalytic domain and the PIF-binding pocket, was further assessed to determine whether either of these regions may have diverged. First, the NCBI Conserved Domain Database (CDD) (Marchler-Bauer et al., 2011) was used to search for a PDK1-like kinase domain (CDD domain cd05581) within each sequence as a measure of overall sequence similarity within the catalytic domain. Second, sequence was classified by the presence or absence of the six highly conserved PIF-binding pocket residues identified in Figure 27. If a putative PDK1 lacked 1) a PDK1-like kinase domain or 2) >2 of the highly conserved PIF-binding pocket residues, that sequence was classified as a more divergent PDK1. The kinase domain of the *Plasmodium vivax* putative PDK1 lacked the first feature, whereas the PIF-binding pocket regions of the *Ciona intestinalis*, *Cryptococcus neoformans*, *Cyanidioschyzon merolae*, *Mucor circinelloides*, *Rhizopus oryzae*, and *Perkinsus marinus* putative PDK1s

```
Plasmodium vivax                ----------------RKYRKDDF-------EI-YMHIGSGNFS---EVFMV-----
Perkinsus marinus               ----------------NTCGFKTY-------DIKVDLLGILDRRIEEISRVS-----
Thalassiosira pseudonana        ----------------RTPSIREFILPSKSSDT-YRPLGEGNFS---TVVIC-----
Fragilariopsis cylindrus        ----------------RPVSIREF-------KK-SETLGIGNFS---EIVIV-----
Phaodactylum tricornutum        ----------------RGVSIREF-------RV-HQELGYGNFS---QIQVV-----
Ectocarpus siliculosus          ----------------PKPSIKDF-------KV-VKELGTGNFS---TIVKA-----
Leishmania major                ----------------TKMAAADF-------EF-GPPLGTGAFS---KVVVG-----
Trypanosoma brucei              ----------------SRMVPADF-------EL-GSALGAGSFS---KVVTA-----
Emiliana huxleyi                ----------------RIFGLHNF-------DA-GGLLGDGNYS---QVIQA-----
Cyanidioschyzon merolae         ----------------QGASRADF-------KA-TELIGEGACS---RVLRA-----
Paramecium tetraurelia          ----------------MNTLNDY-------EI-IEKLGSGSYG---DVMLA-----
Tetrahymena thermophila         ----------------AILRNQLF-------SN-NKNLGRGSFG---EVILA-----
Piriformospora indica           ----------------SSNSAQNY-------QF-QDFLGEGSYS---SVYTA-----
Caenorhabditis briggsae         ----------------PKRSPSDF-------TF-LTSLGEGAYS---QVFRC-----
Caenorhabditis elegans          ----------------PKRTSNDF-------MF-LQSMGEGAYS---QVFRC-----
Coprinopsis cinerea             ----------------QPACLDDF-------DV-GEEIAWGSLA---TIVEG-----
Laccaria bicolor                ----------------RHACLDDF-------LI-GEEIAWGSLA---TIVEA-----
Malassezia globosa              ----------------RRRTTSDF-------VF-GEVLGEGSYS---TVIKAWDVHD
Naegleria gruberi               ----------------SKYTREDF-------DF-LKQLGKGAYS---EVFLT-----
Trichomonas vaginalis           ----------------RQKRKDDF-------NL-GQVIGQGAFG---QVLEV-----
Ustilago maydis                 PTPAAPERATTPSLRASSARGPHDF-------HF-GETLGEGSYS---TVLEAWDLVC
Puccinia graminis               ----------------ARKGIKDF-------IV-GDILGEGSYS---TVYHV-----
Cryptococcus neoformans         ----------------KKHSLDDW-------VL-GEELGVGSYS---TVYCVTPSAN
Entamoeba histolytica           ----------------MAKRENNF-------IF-GDTLGEGSFG---AVILT-----
Saccharomyces cerevisiae        ----------------VKMGIKDF-------KF-GEQLGDGSYS---SVVLA-----
Sporobolomyces roseus           SGSTGASMIPNSMGVVGSKRTVDDF-------EF-GEVLGEGSYS---TVTLV-----
Rhyzopus oryzae                 ----------------QRRTVTDF-------EY-GDILGEGSYS---TVLVG-----
Mucor circinelloides            ----------------HRRIVTDF-------DY-GEILGEGSYS---TVLIG-----
Clonorchis sinensis             ----------------AVPRPSDF-------RF-GKEIGQGSFS---NVYVV-----
Schistosoma mansoni             ----------------HIPKPSDF-------KF-GKEIGHGSFS---TVYVV-----
Phytophthora infestans          ----------------KKPSPADF-------MF-GATLGEGAYA---RVVHA-----
Phytophthora ramorum            ----------------KKPSPADF-------MF-GATLGEGAYA---RVVHA-----
Schizosaccharomyces pombe       ----------------NIKRVSDF-------KF-GEILGEGSYS---TVLTA-----
Ciona intestinalis              ----------------NKKGPDDF-------MV-AKLLGEDEES---TVVLA-----
Dictyostelium discoideum        ----------------KKKTIEDF-------II-GKVLGEGSYG---AVVLG-----
Dictyostelium purpureum         ----------------KKKTIEDF-------II-GKVLGEGSYG---AVVLG-----
Batrachochytrium dendrobatidis  ----------------RKRQTYDF-------KF-GSILGEGSYS---TVLSA-----
Daphnia pulex                   ----------------PKKGPNDF-------IF-GRVLGDGSFS---TVYLA-----
Saitoella complicata            ----------------IKKGVKDF-------QF-GRTLGEGSYS---TVMAA-----
Neurospora crassa               ----------------VKKGVRDF-------TF-GRILGEGSYS---TVYLA-----
Arthroderma otae                ----------------VKKGVRDF-------VF-GRTLGEGSYS---QVVAA-----
Aspergillus terreus             ----------------IKKGVRDF-------SF-GSTLGEGSYS---TVVLG-----
Xanthoria parietina             ----------------VKKGFRDF-------QF-GRTLGEGSYS---TVMAA-----
Cladonia grayi                  ----------------VKKGVKDF-------QF-GRTLGEGSYS---TVIAA-----
Aplysia californica             AATAAANKAR-------VKKTPNDF-------IF-GKVIGEGSYS---TVYLA-----
Gonapodya prolifera             ----------------RRRTANDF-------TF-GDSIGDGSYS---TVVHA-----
Glomus intraradices             ----------------RKKGVKDF-------EF-GKTLGEGSYS---TVVAA-----
Hydra magnipapillata            ----------------IKYKREDF-------IF-GKTLGEGSYS---MVVNA-----
Haemaphysalis longicornis       ----------------AKMSANDF-------IF-GKLIGEGSFS---MVYLA-----
Monosiga brevicollis            ----------------------------------------------------MA-----
Salpingoeca sp. ATCC50818       ----------------RKRRPDDF-------II-SKRLGQGSYS---SVFLC-----
Monodelphis domestica           ----------------WEMRPEDF-------AL-GRILGTGSFA---TVVLA-----
Amphimedon queenslandica        ----------------QKKSREDF-------NF-DRIIGEGSYS---TVILA-----
Patiria pectinifera             ----------------KKKQRQDF-------KF-GKILGEGSYS---EVVLA-----
Strogylocentrotus purpuratus    ----------------LKKKRADF-------RF-GTILGEGSYS---EVVLA-----
Branchiostoma floridae          ----------------RKKTPNDF-------KF-GRILGEGSYS---TVVLA-----
Trichoplax adhaerens            ----------------GKKKAEDF-------EF-GKLIGEGSYS---TVVLA-----
Xenopus laevis                  ----------------RKKRAQDF-------KF-GKILGEGSFS---TVVLA-----
Xenopus tropicalis              ----------------RKKRPQDF-------KF-GKILGEGSFS---TVVLA-----
Anolis carolinensis             ----------------RKKRPEDF-------KF-GKILGEGSFS---TVVLA-----
Gallus gallus                   ----------------RKKRPDDF-------KF-GKILGEGSFS---TVVLA-----
```

Figure 27. Alignment of the PIF-binding pocket region of 100 putative PDK1s. All 9 PIF-binding pocket residues from Frodin et al., 2002 are highlighted. Purple highlight indicates this amino acid binds the phosphate in PIF, and corresponds to K76 and R131 of human PDK1. Blue highlight indicates this amino acid binds the last phenylalanine in PIF, and corresponds to F82, F147, and F149 of human PDK1. Yellow highlight indicates this amino acid binds the first two phenylalanines in PIF, and corresponds to K115, I119, Q150, and L155 of human PDK1. Finally, green highlight indicates the conserved lysine required for catalytic activity in all kinases (not considered a conserved PIF-binding pocket residue here). Species shown in green text have an experimentally confirmed PDK1 (see text for references), species in blue text may have an incomplete PDK1 sequence, and species in red text have a dubious PDK1 due to lacking more than 2 of the 6 highly conserved PIF-binding pocket residues.

```
Taeniopygia guttata             ----------------RKKRPDDF-------KF-GKILGEGSFS---TVVLA-----
Bos taurus                      ----------------RKKRPEDF-------KF-GKILGEGSFS---TVVLA-----
Canis lupus familiaris          ----------------RKKRPEDF-------KF-GKILGEGSFS---TVVLA-----
Homo sapiens                    ----------------RKKRPEDF-------KF-GKILGEGSFS---TVVLA-----
Danio rerio                     ----------------RKKRPEDF-------RF-GKILGEGSFS---TVVLA-----
Dicentrarchus labrax            ----------------RKKKPEDF-------RF-GKILGEGSFS---TVVLA-----
Drosophila melanogaster         ----------------PRRSPNDF-------IF-GRYIGEGSYS---IVYLA-----
Tribolium castaneum             ----------------RKRTSADF-------VF-GKVIGEGSFS---TVYLA-----
Acromyrmex echinatior           ----------------HKRTPKDF-------IF-GKVIGEGSFS---TVYLA-----
Acyrthosiphon pisum             ----------------TKRNPKDY-------IF-GKVIGEGSYS---TVYLA-----
Ostreococcus lucimarinus        ----------------EMCAMRDF-------VT-VDTIGEGSYSSVREVFLA-----
Ostreococcus tauri              ACRLAIERARTPPRT--PPFEISDF-------VT-VDVIGEGSYSDVREVFLS-----
Bigelowiella natans             ----------------KKIGVQDF-------EF-KGLLGEGSYA---KVYLA-----
Micromonas sp. RCC299           ------------------MSHF-------EV-IQELGDGSFS---EVLLC-----
Pinus taeda                     ----------------EPFRIEDF-------EC-EKLHGVGSYS---RVVRA-----
Setaria italica                 ----------------EQFTAADF-------EL-GKIYGVGSYS---KVVRA-----
Sorghum bicolor                 ----------------EQFTADDF-------VL-GKIYGVGSYS---KVVRA-----
Zea mays                        ----------------EQFTADDF-------VL-GKIYGVGSYS---KVVRA-----
Oryza sativa                    ----------------EQFTVGDF-------EL-GKIYGVGSYS---KVVRA-----
Hordeum vulgare                 ----------------EQFTAADF-------EL-GKIYGVGSYS---KVVRA-----
Brachypodium distachyon         ----------------EQFTVDEF-------EL-GRIYGVGSYS---KVVRA-----
Aquilegia coerulea              ----------------ENFTINDF-------QL-GKIYGVGSYS---KVVRA-----
Arabidopsis thaliana            ----------------ENFTSHDF-------EF-GKIYGVGSYS---KVVRA-----
Mimulus guttatus                ----------------ENFSIDDF-------EL-GKIYGVGSYS---KVVRA-----
Cucumis sativus                 ----------------ENFTIQDF-------EL-GKIYGVGSYS---KVVRA-----
Populus trichocarpa             ----------------ENFTIHDF-------EL-GKIYGVGSYS---KVVRA-----
Glycine max                     ----------------ENYTIQDF-------EL-GKIYGVGSYS---KVVRA-----
Phaseolus vulgaris              ----------------ENYTIQDF-------EL-GKIYGVGSYS---KVVRA-----
Citrus sinensis                 ----------------EHFSIQDF-------EL-GKIYGVGSYS---KVVRA-----
Ricinus communis                ----------------ENFSIQDF-------EL-GKIYGVGSYS---KVVRA-----
Solanum lycopersicum            ----------------ENFTIQDF-------EL-GKIYGVGSYS---KVVRA-----
Eucalyptus grandis              ----------------ESFTIQDF-------EL-GKIYGVGSYS---KVVRA-----
Prunus persica                  ----------------EQFSIQDF-------EL-GKIYGVGSYS---KVVRA-----
Vitis vinifera                  ----------------ENFTIQDF-------EL-GKIYGVGSYS---KVVRA-----
Physcomitrella patens           ----------------MDFTSNDF-------LF-AKLLGLGSYS---KVTKA-----
Selaginella moellendorffii      ----------------QPYTYQDF-------AY-GRLLGMGSYS---KVVRA-----
Chlamydomnoas reinhardtii       ----------------VTLTIKDF-------DV-LGRIGDGSFS---TVFLA-----
Volvox carteri                  ----------------VALTIRDF-------HI-LGRIGDGSFS---TVFLA-----

Conserved PIF pocket residue(s) at this position       F
```

Figure 27, continued.

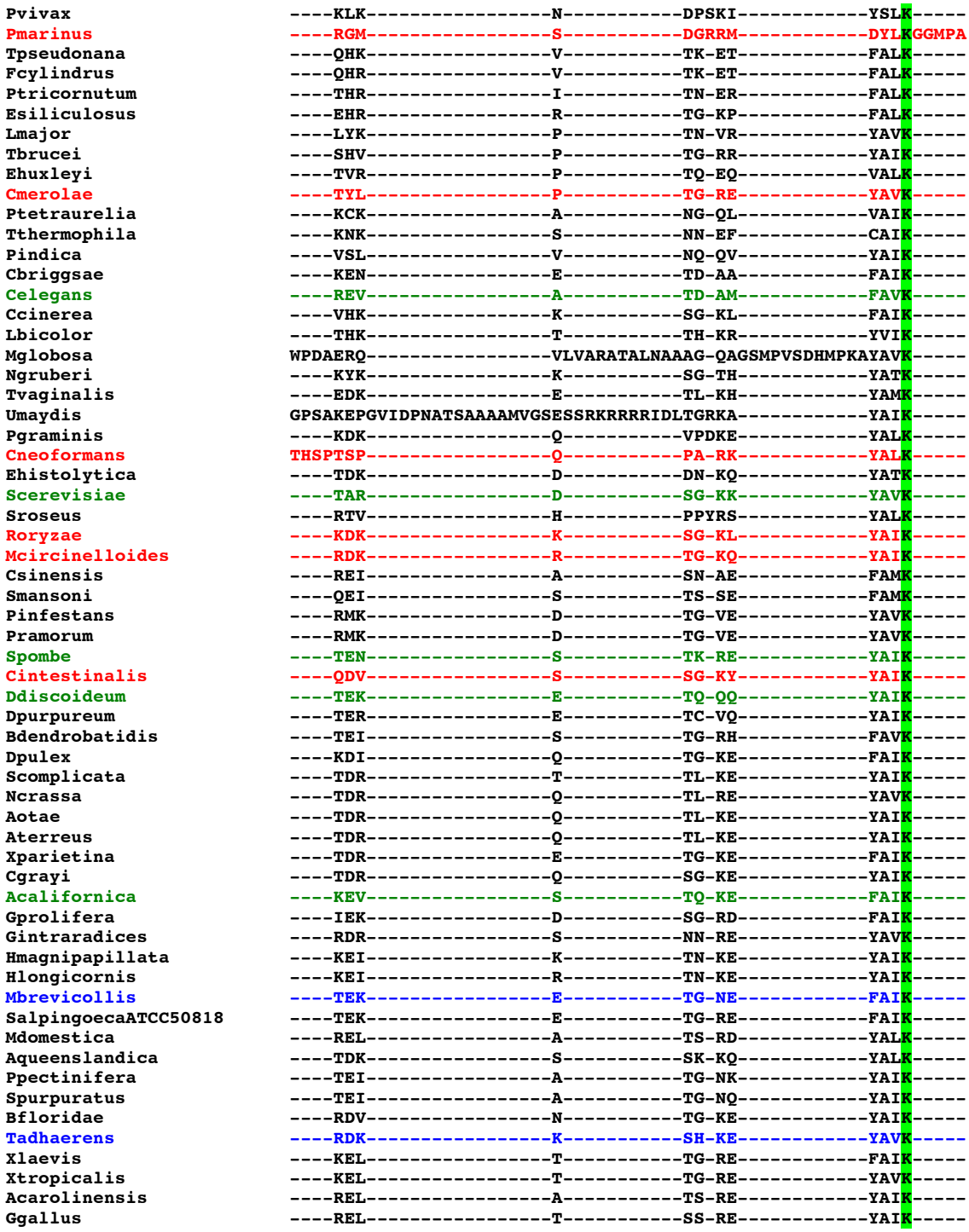


Figure 27, continued.

```
Tguttata                ----REL-----------------A-----------SS-RE-----------YAIK-----
Btaurus                 ----REL-----------------A-----------TS-RE-----------YAIK-----
Clupusfamiliaris        ----REL-----------------A-----------TS-RE-----------YAIK-----
Hsapiens                ----REL-----------------A-----------TS-RE-----------YAIK-----
Drerio                  ----KEH-----------------T-----------TG-KE-----------YAIK-----
Dlabrax                 ----REQ-----------------V-----------TG-KE-----------FAIK-----
Dmelanogaster           ----VDI-----------------H-----------SR-RE-----------YAIK-----
Tcastaneum              ----KDV-----------------H-----------TS-RE-----------FAIK-----
Aechinatior             ----KDI-----------------H-----------TN-KE-----------YAIK-----
Apisum                  ----KDI-----------------H-----------TN-RE-----------HAIK-----
Olucimarinus            ----NKP-----------------T--------------ER-----------YALK-----
Otauri                  ----SRP-----------------S--------------ER-----------YALK-----
Bnatans                 ----QKK-----------------S-----------DQ-CQ-----------YAAK-----
MicromonasRCC299        ----RRK-----------------S-----------DQ-SV-----------CALK-----
Ptaeda                  ----KKK-----------------D-----------TG-KI-----------YALK-----
Sitalica                ----KKK-----------------D-----------TG-NV-----------YALK-----
Sbicolor                ----TKK-----------------D-----------TG-RV-----------YALK-----
Zmays                   ----KKK-----------------D-----------TG-RV-----------YALK-----
Osativa                 ----KKK-----------------D-----------TG-NV-----------YALK-----
Hvulgare                ----RKK-----------------D-----------TG-NV-----------YALK-----
Bdistachyon             ----KKK-----------------D-----------TG-NV-----------YALK-----
Acoerulea               ----KKK-----------------D-----------TG-KV-----------YALK-----
Athaliana               ----KKK-----------------E-----------TG-TV-----------YALK-----
Mguttatus               ----KKK-----------------D-----------TG-IV-----------YAMK-----
Csativus                ----KKK-----------------D-----------TG-IV-----------YALK-----
Ptrichocarpa            ----KKK-----------------D-----------TG-TV-----------YALK-----
Gmax                    ----KKK-----------------D-----------TG-IV-----------YALK-----
Pvulgaris               ----KKK-----------------D-----------TG-TV-----------YALK-----
Csinensis               ----KKI-----------------D-----------TG-TV-----------YALK-----
Rcommunis               ----KKK-----------------D-----------TG-MV-----------YALK-----
Slycopersicum           ----KKK-----------------D-----------TA-NV-----------YALK-----
Egrandis                ----KKK-----------------D-----------TG-TV-----------YALK-----
Ppersica                ----KKK-----------------D-----------TG-TV-----------YALK-----
Vvinifera               ----RKK-----------------D-----------TG-IV-----------YALK-----
Ppatens                 ----KRK-----------------N-----------TG-EI-----------YALK-----
Smoellendorffii         ----KKK-----------------D-----------SG-AE-----------FALK-----
ChlorellaNC64A          ----RHR-----------------A-----------TG-RD-----------YALK-----
Creinhardtii            ----RQK-----------------Q-----------SG-KQ-----------YAIK-----
Vcarteri                ----QQK-----------------Q-----------TG-KQ-----------YAIK-----
                                                                                  *

Conserved PIF pocket residue(s)
```

Figure 27, continued.

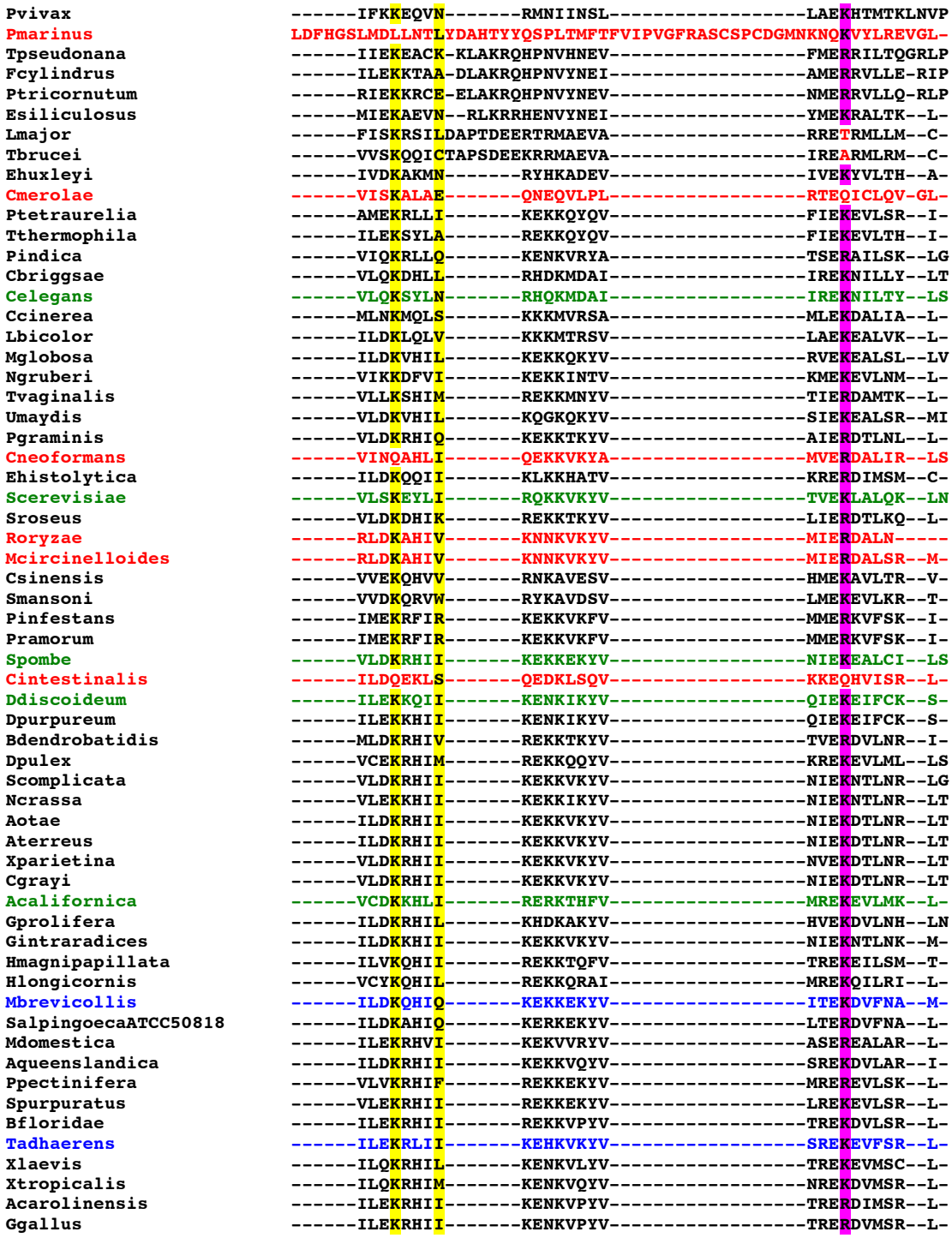


Figure 27, continued.

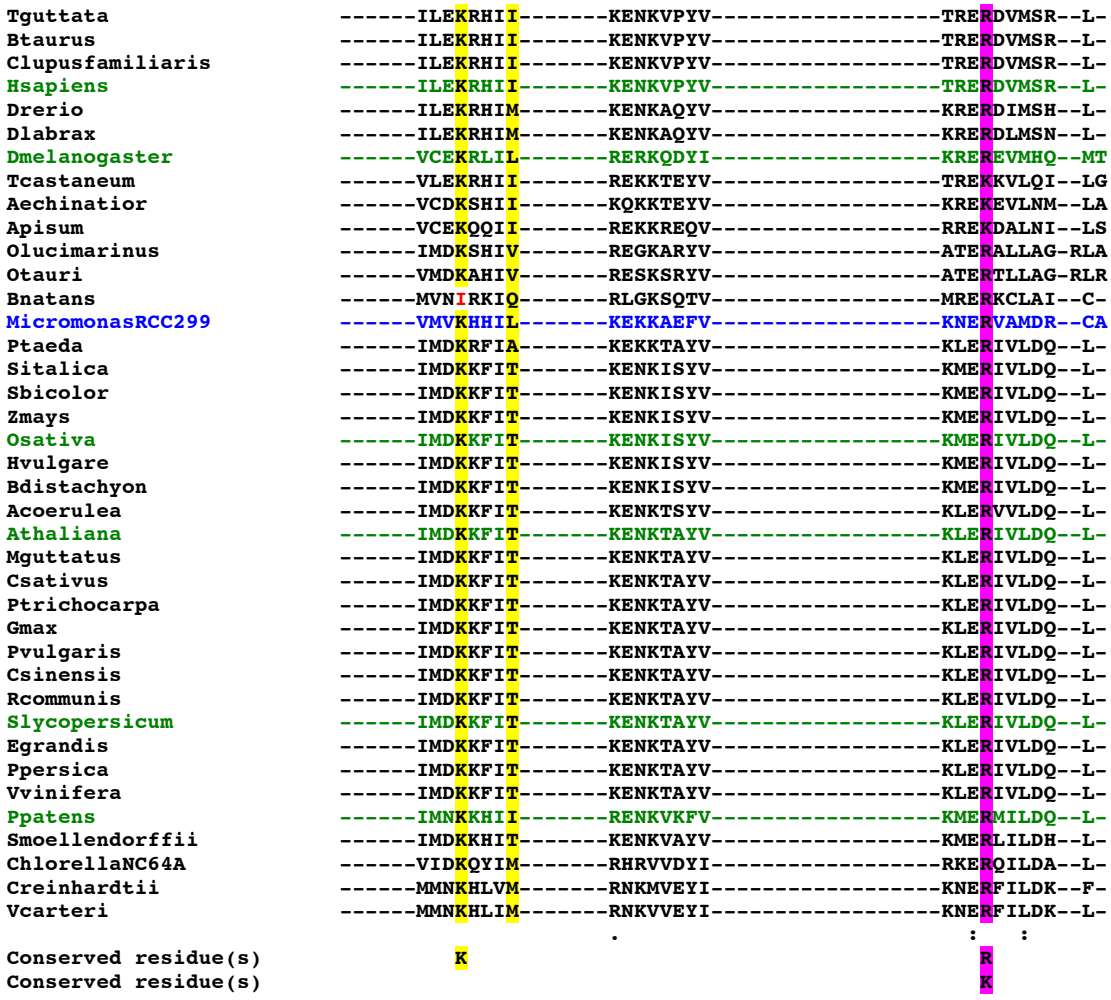


Figure 27, continued.

```
Pvivax                  G-----------------------------------------------------------
Pmarinus                D-----------------------------------------------------------
Tpseudonana             R-----------------------------------------------------------
Fcylindrus              P-----------------------------------------------------------
Ptricornutum            R-----------------------------------------------------------
Esiliculosus            S-----------------------------------------------------------
Lmajor                  E-----------------------------------------------------------
Tbrucei                 S-----------------------------------------------------------
Ehuxleyi                D-----------------------------------------------------------
Cmerolae                G-----------------------------------------------------------
Ptetraurelia            K-----------------------------------------------------------
Tthermophila            R-----------------------------------------------------------
Pindica                 AAN---------------------------------------------------------
Cbriggsae               QTCGG-------------------------------------------------------
Celegans                QECGG-------------------------------------------------------
Ccinerea                SSHP--------------------------------------------------------
Lbicolor                ASRPQ-------------------------------------------------------
Mglobosa                N-----------------------------------------------------------
Ngruberi                S-----------------------------------------------------------
Tvaginalis              N-----------------------------------------------------------
Umaydis                 R-----------------------------------------------------------
Pgraminis               DH----------------------------------------------------------
Cneoformans             DPRPSKGHKRGVSSSSSSGYAQTGSAGKRRSTASIGGQSSMASVSGGTVSNSKKDTRDRL
Ehistolytica            H-----------------------------------------------------------
Scerevisiae             G-----------------------------------------------------------
Sroseus                 DG----------------------------------------------------------
Roryzae                 ----------------------------------------------------------
Mcircinelloides         N-----------------------------------------------------------
Csinensis               N-----------------------------------------------------------
Smansoni                N-----------------------------------------------------------
Pinfestans              S-----------------------------------------------------------
Pramorum                S-----------------------------------------------------------
Spombe                  K-----------------------------------------------------------
Cintestinalis           E-----------------------------------------------------------
Ddiscoideum             N-----------------------------------------------------------
Dpurpureum              N-----------------------------------------------------------
Bdendrobatidis          H-----------------------------------------------------------
Dpulex                  DKVKT-------------------------------------------------------
Scomplicata             D-----------------------------------------------------------
Ncrassa                 E-----------------------------------------------------------
Aotae                   D-----------------------------------------------------------
Aterreus                E-----------------------------------------------------------
Xparietina              E-----------------------------------------------------------
Cgrayi                  D-----------------------------------------------------------
Acalifornica            D-----------------------------------------------------------
Gprolifera              G-----------------------------------------------------------
Gintraradices           N-----------------------------------------------------------
Hmagnipapillata         N-----------------------------------------------------------
Hlongicornis            SARP--------------------------------------------------------
Mbrevicollis            D-----------------------------------------------------------
SalpingoecaATCC50818    R-----------------------------------------------------------
Mdomestica              D-----------------------------------------------------------
Aqueenslandica          D-----------------------------------------------------------
Ppectinifera            D-----------------------------------------------------------
Spurpuratus             D-----------------------------------------------------------
Bfloridae               N-----------------------------------------------------------
Tadhaerens              N-----------------------------------------------------------
Xlaevis                 D-----------------------------------------------------------
Xtropicalis             D-----------------------------------------------------------
Acarolinensis           D-----------------------------------------------------------
Ggallus                 D-----------------------------------------------------------
```

Figure 27, continued.

```
Tguttata                D-----------------------------------------------------
Btaurus                 D-----------------------------------------------------
Clupusfamiliaris        D-----------------------------------------------------
Hsapiens                D-----------------------------------------------------
Drerio                  D-----------------------------------------------------
Dlabrax                 D-----------------------------------------------------
Dmelanogaster           N-----------------------------------------------------
Tcastaneum              SS----------------------------------------------------
Aechinatior             DAK---------------------------------------------------
Apisum                  NSG---------------------------------------------------
Olucimarinus            D-----------------------------------------------------
Otauri                  E-----------------------------------------------------
Bnatans                 D-----------------------------------------------------
MicromonasRCC299        D-----------------------------------------------------
Ptaeda                  D-----------------------------------------------------
Sitalica                D-----------------------------------------------------
Sbicolor                D-----------------------------------------------------
Zmays                   D-----------------------------------------------------
Osativa                 D-----------------------------------------------------
Hvulgare                D-----------------------------------------------------
Bdistachyon             D-----------------------------------------------------
Acoerulea               D-----------------------------------------------------
Athaliana               E-----------------------------------------------------
Mguttatus               D-----------------------------------------------------
Csativus                D-----------------------------------------------------
Ptrichocarpa            D-----------------------------------------------------
Gmax                    D-----------------------------------------------------
Pvulgaris               D-----------------------------------------------------
Csinensis               D-----------------------------------------------------
Rcommunis               D-----------------------------------------------------
Slycopersicum           D-----------------------------------------------------
Egrandis                D-----------------------------------------------------
Ppersica                D-----------------------------------------------------
Vvinifera               D-----------------------------------------------------
Ppatens                 D-----------------------------------------------------
Smoellendorffii         D-----------------------------------------------------
ChlorellaNC64A          Q-----------------------------------------------------
Creinhardtii            D-----------------------------------------------------
Vcarteri                D-----------------------------------------------------

Conserved residue(s)
```

Figure 27, continued.

```
Pvivax                  ------------------------------------------------------------
Pmarinus                ------------------------------------------------------------
Tpseudonana             ------------------------------------------------------------
Fcylindrus              ------------------------------------------------------------
Ptricornutum            ------------------------------------------------------------
Esiliculosus            ------------------------------------------------------------
Lmajor                  ------------------------------------------------------------
Tbrucei                 ------------------------------------------------------------
Ehuxleyi                ------------------------------------------------------------
Cmerolae                ------------------------------------------------------------
Ptetraurelia            ------------------------------------------------------------
Tthermophila            ------------------------------------------------------------
Pindica                 ------------------------------------------------------------
Cbriggsae               ------------------------------------------------------------
Celegans                ------------------------------------------------------------
Ccinerea                ------------------------------------------------------------
Lbicolor                ------------------------------------------------------------
Mglobosa                ------------------------------------------------------------
Ngruberi                ------------------------------------------------------------
Tvaginalis              ------------------------------------------------------------
Umaydis                 ------------------------------------------------------------
Pgraminis               ------------------------------------------------------------
Cneoformans             SIVTTSSAASSPVLTASSGSTQLSPTAEQTEMLIRGGEDGKDGQDGQETPSREWDRDRDW
Ehistolytica            ------------------------------------------------------------
Scerevisiae             ------------------------------------------------------------
Sroseus                 ------------------------------------------------------------
Roryzae                 ------------------------------------------------------------
Mcircinelloides         ------------------------------------------------------------
Csinensis               ------------------------------------------------------------
Smansoni                ------------------------------------------------------------
Pinfestans              ------------------------------------------------------------
Pramorum                ------------------------------------------------------------
Spombe                  ------------------------------------------------------------
Cintestinalis           ------------------------------------------------------------
Ddiscoideum             ------------------------------------------------------------
Dpurpureum              ------------------------------------------------------------
Bdendrobatidis          ------------------------------------------------------------
Dpulex                  ------------------------------------------------------------
Scomplicata             ------------------------------------------------------------
Ncrassa                 ------------------------------------------------------------
Aotae                   ------------------------------------------------------------
Aterreus                ------------------------------------------------------------
Xparietina              ------------------------------------------------------------
Cgrayi                  ------------------------------------------------------------
Acalifornica            ------------------------------------------------------------
Gprolifera              ------------------------------------------------------------
Gintraradices           ------------------------------------------------------------
Hmagnipapillata         ------------------------------------------------------------
Hlongicornis            ------------------------------------------------------------
Mbrevicollis            ------------------------------------------------------------
SalpingoecaATCC50818    ------------------------------------------------------------
Mdomestica              ------------------------------------------------------------
Aqueenslandica          ------------------------------------------------------------
Ppectinifera            ------------------------------------------------------------
Spurpuratus             ------------------------------------------------------------
Bfloridae               ------------------------------------------------------------
Tadhaerens              ------------------------------------------------------------
Xlaevis                 ------------------------------------------------------------
Xtropicalis             ------------------------------------------------------------
Acarolinensis           ------------------------------------------------------------
Ggallus                 ------------------------------------------------------------
```

Figure 27, continued.

```
Tguttata                ------------------------------------------------------------
Btaurus                 ------------------------------------------------------------
Clupusfamiliaris        ------------------------------------------------------------
Hsapiens                ------------------------------------------------------------
Drerio                  ------------------------------------------------------------
Dlabrax                 ------------------------------------------------------------
Dmelanogaster           ------------------------------------------------------------
Tcastaneum              ------------------------------------------------------------
Aechinatior             ------------------------------------------------------------
Apisum                  ------------------------------------------------------------
Olucimarinus            ------------------------------------------------------------
Otauri                  ------------------------------------------------------------
Bnatans                 ------------------------------------------------------------
MicromonasRCC299        ------------------------------------------------------------
Ptaeda                  ------------------------------------------------------------
Sitalica                ------------------------------------------------------------
Sbicolor                ------------------------------------------------------------
Zmays                   ------------------------------------------------------------
Osativa                 ------------------------------------------------------------
Hvulgare                ------------------------------------------------------------
Bdistachyon             ------------------------------------------------------------
Acoerulea               ------------------------------------------------------------
Athaliana               ------------------------------------------------------------
Mguttatus               ------------------------------------------------------------
Csativus                ------------------------------------------------------------
Ptrichocarpa            ------------------------------------------------------------
Gmax                    ------------------------------------------------------------
Pvulgaris               ------------------------------------------------------------
Csinensis               ------------------------------------------------------------
Rcommunis               ------------------------------------------------------------
Slycopersicum           ------------------------------------------------------------
Egrandis                ------------------------------------------------------------
Ppersica                ------------------------------------------------------------
Vvinifera               ------------------------------------------------------------
Ppatens                 ------------------------------------------------------------
Smoellendorffii         ------------------------------------------------------------
ChlorellaNC64A          ------------------------------------------------------------
Creinhardtii            ------------------------------------------------------------
Vcarteri                ------------------------------------------------------------

Conserved residue(s)
```

Figure 27, continued.

```
Pvivax                  ------------------------------------------------------------
Pmarinus                ------------------------------------------------------------
Tpseudonana             ------------------------------------------------------------
Fcylindrus              ------------------------------------------------------------
Ptricornutum            ------------------------------------------------------------
Esiliculosus            ------------------------------------------------------------
Lmajor                  ------------------------------------------------------------
Tbrucei                 ------------------------------------------------------------
Ehuxleyi                ------------------------------------------------------------
Cmerolae                ------------------------------------------------------------
Ptetraurelia            ------------------------------------------------------------
Tthermophila            ------------------------------------------------------------
Pindica                 ------------------------------------------------------------
Cbriggsae               ------------------------------------------------------------
Celegans                ------------------------------------------------------------
Ccinerea                ------------------------------------------------------------
Lbicolor                ------------------------------------------------------------
Mglobosa                ------------------------------------------------------------
Ngruberi                ------------------------------------------------------------
Tvaginalis              ------------------------------------------------------------
Umaydis                 ------------------------------------------------------------
Pgraminis               ------------------------------------------------------------
Cneoformans             DNMTRSRPPSPVREESAEGGEKEKDEEERSGAEPVELGAAIHLTLPPPQIPSTPEPRGSP
Ehistolytica            ------------------------------------------------------------
Scerevisiae             ------------------------------------------------------------
Sroseus                 ------------------------------------------------------------
Roryzae                 ------------------------------------------------------------
Mcircinelloides         ------------------------------------------------------------
Csinensis               ------------------------------------------------------------
Smansoni                ------------------------------------------------------------
Pinfestans              ------------------------------------------------------------
Pramorum                ------------------------------------------------------------
Spombe                  ------------------------------------------------------------
Cintestinalis           ------------------------------------------------------------
Ddiscoideum             ------------------------------------------------------------
Dpurpureum              ------------------------------------------------------------
Bdendrobatidis          ------------------------------------------------------------
Dpulex                  ------------------------------------------------------------
Scomplicata             ------------------------------------------------------------
Ncrassa                 ------------------------------------------------------------
Aotae                   ------------------------------------------------------------
Aterreus                ------------------------------------------------------------
Xparietina              ------------------------------------------------------------
Cgrayi                  ------------------------------------------------------------
Acalifornica            ------------------------------------------------------------
Gprolifera              ------------------------------------------------------------
Gintraradices           ------------------------------------------------------------
Hmagnipapillata         ------------------------------------------------------------
Hlongicornis            ------------------------------------------------------------
Mbrevicollis            ------------------------------------------------------------
SalpingoecaATCC50818    ------------------------------------------------------------
Mdomestica              ------------------------------------------------------------
Aqueenslandica          ------------------------------------------------------------
Ppectinifera            ------------------------------------------------------------
Spurpuratus             ------------------------------------------------------------
Bfloridae               ------------------------------------------------------------
Tadhaerens              ------------------------------------------------------------
Xlaevis                 ------------------------------------------------------------
Xtropicalis             ------------------------------------------------------------
Acarolinensis           ------------------------------------------------------------
Ggallus                 ------------------------------------------------------------
```

Figure 27, continued.

```
Tguttata                ------------------------------------------------------------
Btaurus                 ------------------------------------------------------------
Clupusfamiliaris        ------------------------------------------------------------
Hsapiens                ------------------------------------------------------------
Drerio                  ------------------------------------------------------------
Dlabrax                 ------------------------------------------------------------
Dmelanogaster           ------------------------------------------------------------
Tcastaneum              ------------------------------------------------------------
Aechinatior             ------------------------------------------------------------
Apisum                  ------------------------------------------------------------
Olucimarinus            ------------------------------------------------------------
Otauri                  ------------------------------------------------------------
Bnatans                 ------------------------------------------------------------
MicromonasRCC299        ------------------------------------------------------------
Ptaeda                  ------------------------------------------------------------
Sitalica                ------------------------------------------------------------
Sbicolor                ------------------------------------------------------------
Zmays                   ------------------------------------------------------------
Osativa                 ------------------------------------------------------------
Hvulgare                ------------------------------------------------------------
Bdistachyon             ------------------------------------------------------------
Acoerulea               ------------------------------------------------------------
Athaliana               ------------------------------------------------------------
Mguttatus               ------------------------------------------------------------
Csativus                ------------------------------------------------------------
Ptrichocarpa            ------------------------------------------------------------
Gmax                    ------------------------------------------------------------
Pvulgaris               ------------------------------------------------------------
Csinensis               ------------------------------------------------------------
Rcommunis               ------------------------------------------------------------
Slycopersicum           ------------------------------------------------------------
Egrandis                ------------------------------------------------------------
Ppersica                ------------------------------------------------------------
Vvinifera               ------------------------------------------------------------
Ppatens                 ------------------------------------------------------------
Smoellendorffii         ------------------------------------------------------------
ChlorellaNC64A          ------------------------------------------------------------
Creinhardtii            ------------------------------------------------------------
Vcarteri                ------------------------------------------------------------

Conserved residue(s)
```

Figure 27, continued.

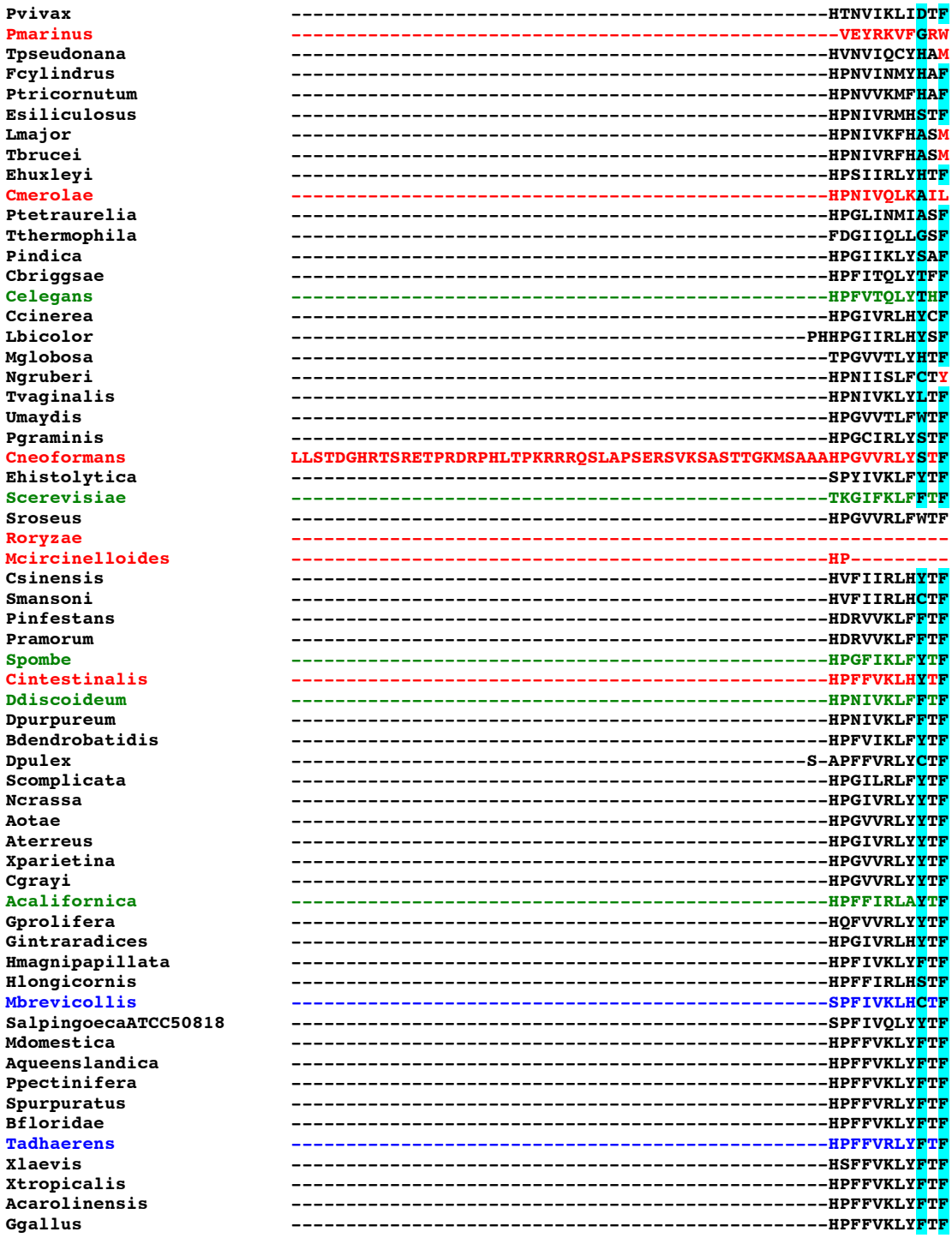


Figure 27, continued.

```
Tguttata                ------------------------------------------------HPFFVKLYFTF
Btaurus                 ------------------------------------------------HPFFVKLYFTF
Clupusfamiliaris        ------------------------------------------------HPFFVKLYFTF
Hsapiens                ------------------------------------------------HPFFVKLYFTF
Drerio                  ------------------------------------------------HPFFVKLYFTF
Dlabrax                 ------------------------------------------------HPFFVKLYFTF
Dmelanogaster           ------------------------------------------------VPGFVNLSCTF
Tcastaneum              ------------------------------------------------CKYFVHLYYTF
Aechinatior             ------------------------------------------------HS-FVRLFCTF
Apisum                  ------------------------------------------------SSLFVKLYCTF
Olucimarinus            ------------------------------------------------CDCVAALRFTF
Otauri                  ------------------------------------------------CEHVARLMFTF
Bnatans                 ------------------------------------------------HPNIVKLHFSF
MicromonasRCC299        ------------------------------------------------VPGVVRLRFTF
Ptaeda                  ------------------------------------------------HPGIIKLYFTF
Sitalica                ------------------------------------------------HPGVIRLFFTF
Sbicolor                ------------------------------------------------HPGVIRLFFTF
Zmays                   ------------------------------------------------HPGVIRLFFTF
Osativa                 ------------------------------------------------HPGVIRLFFTF
Hvulgare                ------------------------------------------------HPGVIRLFFTF
Bdistachyon             ------------------------------------------------HPGVIRLFFTF
Acoerulea               ------------------------------------------------HPGIVRLFFTF
Athaliana               ------------------------------------------------HPGIIKLYFTF
Mguttatus               ------------------------------------------------HPGVVRLFFTF
Csativus                ------------------------------------------------HPGVVRLFFTF
Ptrichocarpa            ------------------------------------------------HPGIVRLFFTF
Gmax                    ------------------------------------------------HPGIVRLYFTF
Pvulgaris               ------------------------------------------------HPGIVRLYFTF
Csinensis               ------------------------------------------------HPGVVRLFFTF
Rcommunis               ------------------------------------------------HPGIVRLFFTF
Slycopersicum           ------------------------------------------------HPGVVRLFFTF
Egrandis                ------------------------------------------------HPGIVRLYFTF
Ppersica                ------------------------------------------------HPGIVRLFFTF
Vvinifera               ------------------------------------------------HPGIVRLFFTF
Ppatens                 ------------------------------------------------HPGVVKLCFTF
Smoellendorffii         ------------------------------------------------HPGVVRLFFTF
ChlorellaNC64A          ------------------------------------------------YDGIAKLYFTF
Creinhardtii            ------------------------------------------------DAGIAKLHFTF
Vcarteri                ------------------------------------------------DAGIAKLHFTF

Conserved residue(s)                                                              F
```

Figure 27, continued.

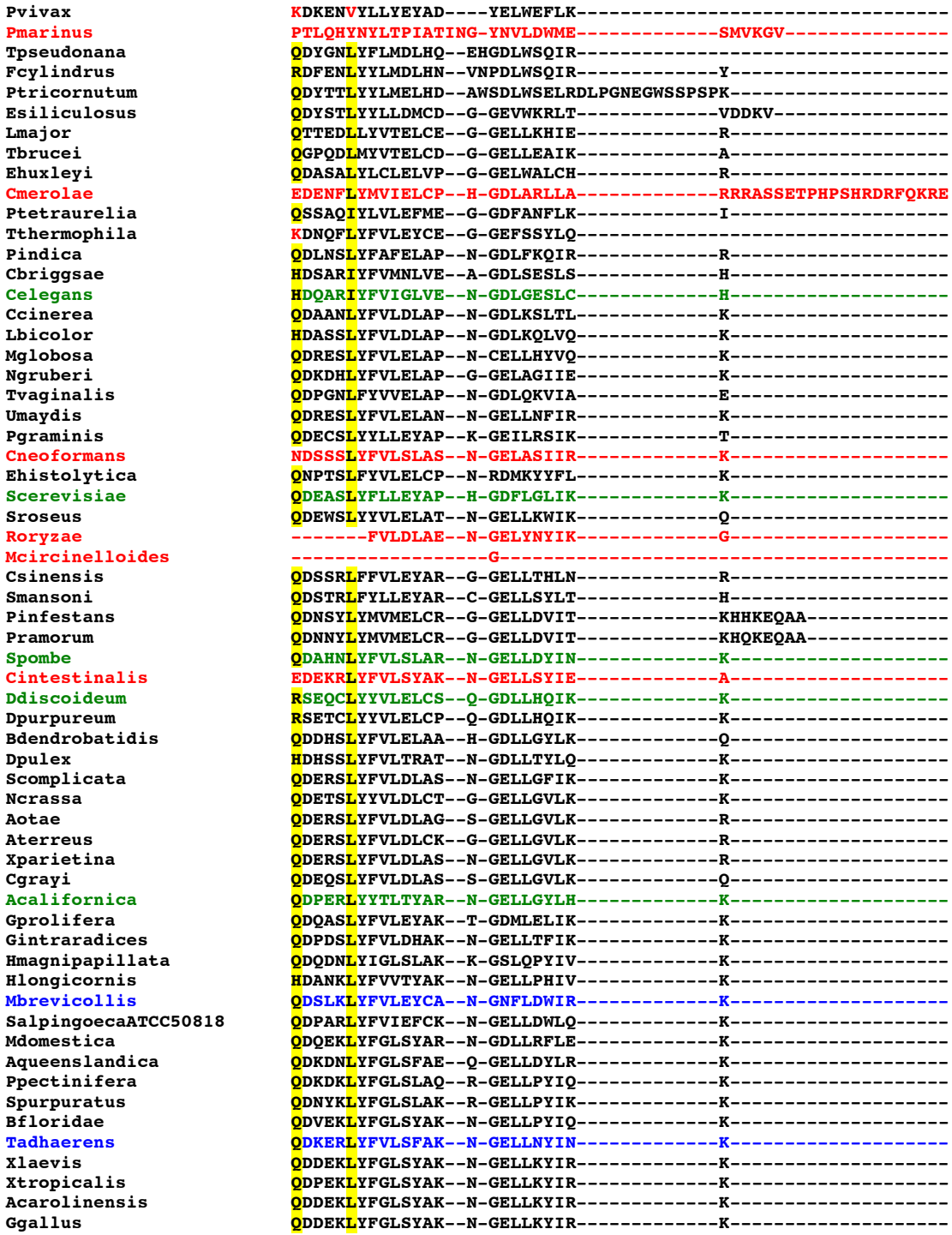


Figure 27, continued.

```
Tguttata                QDDEKLYFGLSYAK--N-GELLKYIR-------------K--------------------
Btaurus                 QDDEKLYFGLSYAK--N-GELLKYIR-------------K--------------------
Clupusfamiliaris        QDDEKLYFGLSYAK--N-GELLKYIR-------------K--------------------
Hsapiens                QDDEKLYFGLSYAK--N-GELLKYIR-------------K--------------------
Drerio                  QDEEKLYFGLSYAK--N-GELLKYIR-------------K--------------------
Dlabrax                 QDDEKLYFGLSYAK--N-GELLKYIR-------------K--------------------
Dmelanogaster           QDQRSLYFVMTYAR--K-GDMLPYIN-------------R--------------------
Tcastaneum              QDTDRLYFVLTYAK--N-GELLQQIN-------------K--------------------
Aechinatior             QDAERLYFVMSYAK--N-GELLPHIN-------------K--------------------
Apisum                  QDTERLYFVMSYAK--N-GDLLPYIN-------------K--------------------
Olucimarinus            QDTYSLYLGMELCT--G-GDLYSQLK-------------R--------------------
Otauri                  QDTYSLYMGFELCP--G-GDLFWQLK-------------R--------------------
Bnatans                 RDTAHLYFVLELIS--G-GELFELIV-------------R--------------------
MicromonasRCC299        QDCNSLYMGMDYCP--G-GELFDQIR-------------R--------------------
Ptaeda                  QDACNIYLGLESCD--G-GELFDQIS-------------R--------------------
Sitalica                QDTYSLYMALESCE--G-GELFDQII-------------R--------------------
Sbicolor                QDTYSLYMALESCE--G-GELFDQIV-------------R--------------------
Zmays                   QDTYSLYMALESCE--G-GELFDQIV-------------R--------------------
Osativa                 QDTYSLYMALESCE--G-GELFDQIV-------------R--------------------
Hvulgare                QDSYSLYMALESCE--G-GELFDQIV-------------R--------------------
Bdistachyon             QDTYSLYMALESCE--G-GELFDQII-------------R--------------------
Acoerulea               QDSFSLYMALESCE--G-GELFDQIT-------------R--------------------
Athaliana               QDTSSLYMALESCE--G-GELFDQIT-------------R--------------------
Mguttatus               QDSFSLYMALESCE--G-GELFDQIT-------------S--------------------
Csativus                QDTFSLYMALESCE--G-GELFDQIT-------------R--------------------
Ptrichocarpa            QDNYSLYMALESCE--G-GELFDQIT-------------R--------------------
Gmax                    QDSFSLYMALESCE--G-GELFDQIT-------------R--------------------
Pvulgaris               QDSFSLYMALESCE--G-GELFDQIT-------------R--------------------
Csinensis               QDTFSLYMALESCE--G-GELFDQIT-------------S--------------------
Rcommunis               QDSFSLYMALESCE--G-GELFDQIT-------------R--------------------
Slycopersicum           QDTFSLYMALESCE--G-GELFDQIT-------------R--------------------
Egrandis                QDTFSLYMALESCE--G-GELFDQIT-------------R--------------------
Ppersica                QDSFSLYMALESCE--G-GELFDQIT-------------R--------------------
Vvinifera               QDTFSLYMALESCE--G-GELFDQIT-------------R--------------------
Ppatens                 QDVHSLYMGLECCT--G-GELFEQIR-------------R--------------------
Smoellendorffii         QDTHNLYMGLECCH--G-GELFDQIR-------------R--------------------
ChlorellaNC64A          QDAYSLYLGLEYCP--N-GELYDQIR-------------L--------------------
Creinhardtii            QDPDNLYMGMEYCA--G-GELYEQIN-------------K--------------------
Vcarteri                QDPNNLYMGMEYCA--G-GELYEQIK-------------R--------------------

Conserved residue(s)    Q    L
Conserved residue(s)    R    I
Conserved residue(s)    H
```

Figure 27, continued.

lacked the second feature. Thus, all 7 of these sequences are shown in Figure 28 as being more divergent than the other putative PDK1s. However, it is necessary to reiterate that the best test of a PDK1 is its ability to phosphorylate AGC kinase substrates in the activation loop. Most of the putative PDK1s investigated here share a good deal of similarity within the catalytic domain and PIF-binding pocket, but the lack of a particular conserved sequence within a putative PDK1 does not necessarily mean it lacks PDK1 function.

4.3c Analysis of PDK1 lipid-binding domain sequences

The presence or absence of a conserved lipid-binding domain was also investigated by CDD search. Two potential lipid-binding domains, the PDK1-like PH domain (CDD domain cd01262) and the PH-like domain (CDD superfamily cl00273), were identified in 47 and 14 putative PDK1s, respectively (Fig. 28). Interestingly, a FYVE domain (CDD domain cd00065) was identified in putative PDK1s from both *Leishmania major* and *Trypanosoma brucei*, but not in any other sequences analyzed (Fig. 28). Finally, a region with very weak similarity (E-value ~10^{-3}) to the PH-like domain was identified in the putative PDK1s of four fungal species: *Malassezia globosa*, *Puccinia graminis*, *Saitoella complicata*, and *Ustilago maydis* (Fig. 28). Because the similarity of this region to other PH and PH-like domains is so low, these proteins were classified as lacking a readily identifiable lipid-binding domain, for a total of 37 putative PDK1s without a conserved lipid-binding domain and 63 putative PDK1s possessing a PH, PH-like, or FYVE domain (Fig. 28). It is important to note that the lipid binding

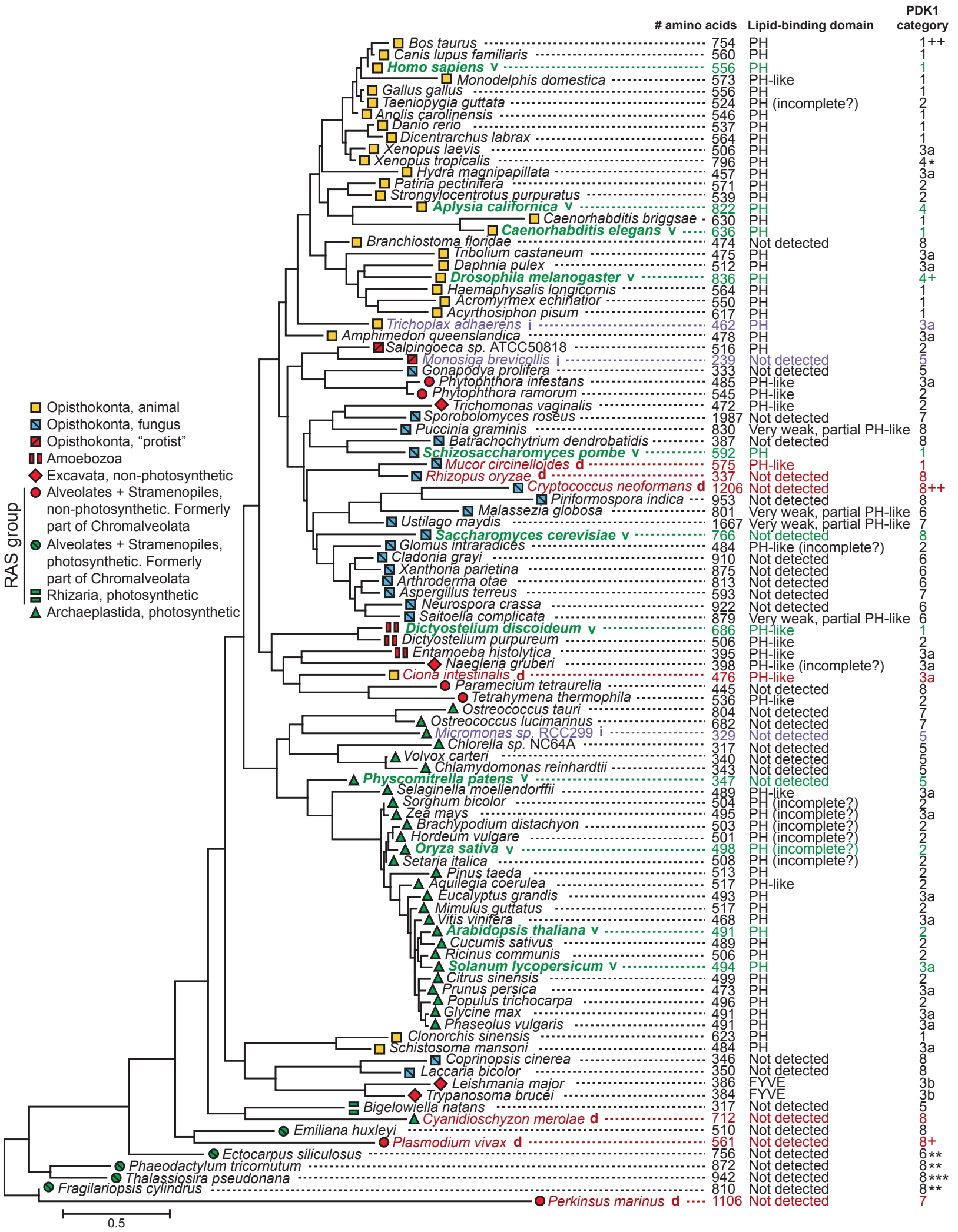


Figure 28. Phylogenetic relationship of putative PDK1 proteins from 100 different eukaryotes. NCBI Conserved Domain Database (Marchler-Bauer et al., 2011) was used to search for protein domains, including the PDK1-like kinase domain, the PDK1-like PH domain, the PH-like domain, and the FYVE domain. A "v" next to the species name indicates species with experimentally verified PDK1; "d" indicates species with potentially dubious PDK1; "i" indicates species whose PDK1 may be incomplete. A + next to the PDK1 category number indicates an insertion within the kinase domain of <150 amino acids; ++ indicates an insertion of >150 amino acids; * indicates the presence of a putative chromo domain outside the kinase domain; ** indicates a putative tetratricopeptide repeat (TPR) domain; *** indicates a putative TPR-like domain

capability of each protein should be experimentally investigated, as not all PH domains mediate lipid interactions (Yu et al., 2004). In contrast, the *S. cerevisiae* PDK1 homologues Pkh1 and Pkh2 do not possess readily identifiable lipid-binding domains but are nevertheless regulated by sphingoid bases (Friant et al., 2001), and because PH domain sequences are less conserved than their folded structure (van Leeuwen et al., 2004) they might not be identified by sequence similarity. Thus, a PH domain does not necessitate phospholipid binding, and lack of a detectable PH domain may not imply lack of regulation by lipids.

4.4 Potential lipid-binding interactions of putative PDK1s

4.4a PDK1 regulation by lipids may be less common than expected

In agreement with a previous report (Deak et al., 1999), qualitative *in vitro* lipid-binding assays have found that PDK1s from both *A. thaliana* and tomato strongly interact with phosphorylated phosphoinositides including PtdIns3P, PtdIns(4,5)P_2, and PA. However, the *P. patens* PDK1 lacks a detectable lipid-binding domain and accordingly does not strongly bind either phospholipids or sphingolipids (Dittrich and Devarenne, 2012). A search for PDK1s from divergent photosynthetic and non-photosynthetic organisms recovered 37 putative PDK1 proteins lacking any strongly identified lipid interaction domains (Fig. 28; Fig. 29). This observation raises the possibility that lipid regulation of PDK1 is not nearly as widespread as might be expected based on published PDK1 sequences. A lack of lipid regulation seems most likely in the 7 very small putative PDK1s I found whose protein domain structure kinase domain (category 5 in Fig. 29). The majority of putative PDK1s without a known

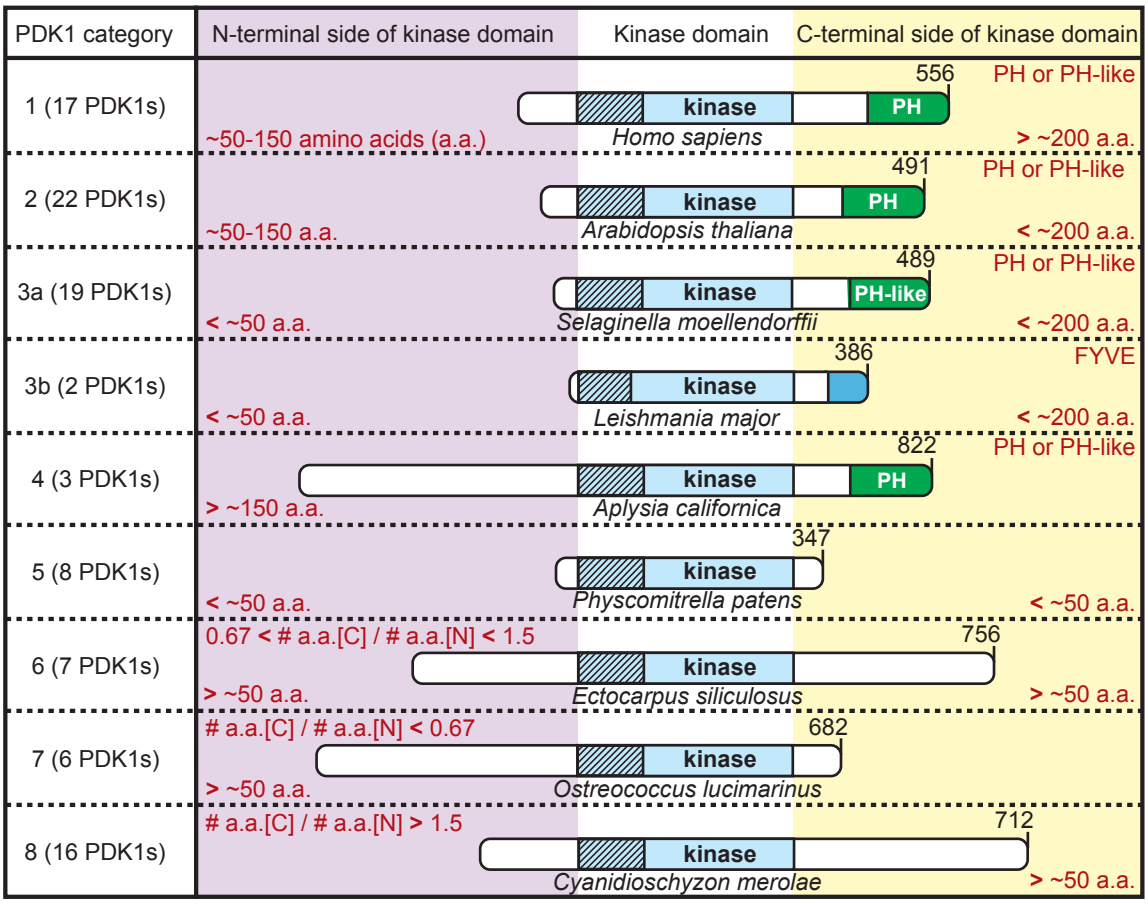


Figure 29. Categorization of putative PDK1s by protein domain structure. For ease of visualization, PDK1 sequences from Figure 1 were divided into categories using three criteria: first, the presence or absence of a lipid-binding domain identified by CDD; second, the number of amino acids on each side of the kinase domain; third, the ratio of amino acids on the C-terminal side of the kinase domain (# a.a.[C]) to amino acids on the N-terminal side of the kinase domain (# a.a. [N]). The left column shows the number of each category (1-8), indicating in parentheses the number of PDK1 sequences from Figure 1 belonging to that category. The right column contains diagrams of one representative PDK1 from each category, including the locations of the kinase domain, PIF-binding pocket (box with diagonal lines), and putative lipid-binding domain (if any) within each protein. Distinguishing characteristics of each category are found in the corners of each section of the right column: the upper-right corner shows the identified lipid-binding domain (if any); the lower-right corner shows the number of amino acids on the C-terminal side of the kinase domain; the lower-left corner shows the number of amino acids on the N-terminal side of the kinase domain; the upper-left corner shows the ratio of # a.a.[C] to # a.a.[N]. If no text is present in a given corner, then that characteristic was not used to distinguish PDK1s found in that category.

resembles the *P. patens* PDK1, with fewer than ~50 amino acids on each side of the lipid-binding domain have more than ~50 amino acids on one or both sides of the kinase domain (categories 6-8 in Fig. 29). A more intriguing possibility for some of these proteins, like the *S. cerevisiae* PDK1 homologue (Casamayor et al., 1999; Friant et al., 2001), is that cryptic lipid- or protein-interaction domains may exist for regulating PDK1 activity and/or localization. Sequences outside the kinase domain could also comprise additional auto-regulatory modules, as the N-terminal region of protein kinase C does (Pearce et al., 2010).

4.4b PDK1 lipid-binding trends in Opisthokonta and Archaeplastida

From the proteins discussed in this chapter, a few general trends seem apparent regarding PDK1 lipid-binding capability. First, the majority of putative PDK1s with a PH or PH-like domain are found in animals and vascular plants (Fig. 28). Only 1 putative PDK1 lacking a detectable lipid-binding domain (from the lancelet *Branchiostoma floridae*) was identified from 50 species of animals and vascular plants (Fig. 28). This suggests that lipid regulation of PDK1 may be a characteristic feature of organisms in both clades. Second, putative PDK1s lacking any detectable lipid-binding domain most often belong to fungi and non-vascular members of the Archaeplastida (Fig. 28). No putative PDK1s with an obvious lipid-binding domain were identified from 8 nonvascular Archaeplastidal species, again suggesting that this may be the most common situation in Archaeplastida, though more species must be analyzed to verify this trend. Only 3 of 21 fungal species possess a readily identifiable PH or PH-like domain within their putative PDK1: *Mucor circinelloides* (belonging to

Mucoromycotina), and *Glomus intraradices* (belonging to Glomeromycota), and the relatively unrelated yeast *Schizosaccharomyces pombe* (an ascomycete belonging to Taphrinomycotina) (Grigoriev et al., 2012). Due to the small number of species discussed here we cannot confidently assert that fungal PDK1s are more likely to lack a PH domain, though this does seem to be the trend to date.

4.4c Lipid-binding trends in RAS, Excavata, and Amoebozoa

Because very few genomes are available from representatives of the RAS group (6 photosynthetic + 6 non-photosynthetic), Excavates (4 non-photosynthetic), and Amoebozoa (3 non-photosynthetic), trends regarding the lipid-binding abilities of these PDK1s are difficult to ascertain, and trends regarding the lipid-binding abilities of these PDK1s are more difficult to determine and less likely to remain true in the future. All 7 species of Amoebozoa and Excavata have putative lipid-binding domains in their PDK1s. Two of the mitochondriate Excavates (*L. major* and *T. brucei*) have a FYVE domain at the C-terminus of the putative PDK1, possibly reflecting a PDK1 modification that occurred only within the ancestor of these organisms, since no other PDK1s investigated have a similar lipid-binding domain (Fig. 28). Interestingly, a draft kinome for *L. major* identified 3 putative PDK1 homologues, 1 with a FYVE domain (used in our analysis) and 2 lacking any obvious lipid-binding domain (Manning et al., 2011). This is discussed further below.

Lipid-binding sequences are present within several unexpected RAS group PDK1s. For example, reasonably close relatives of diatoms and brown algae, the oomycetes *Phytophthora infestans* and *Phytophthora ramorum*, have putative PDK1s

that appear more closely related to animal PDK1s and have PH-like domains. A second example comes from the ciliates *Paramecium tetraurelia* and *Tetrahymena thermophila*, only 1 of which seems to have a PH-like domain. PDK1s from 3 diatoms (*Phaeodactylum tricornutum*, *Thalassiosira pseudonana*, and *Fragilariopsis cylindrus*) and 1 brown alga (*Ectocarpus siliculosus*) lack a PH-like domain but have putative tetratricopeptide repeat (TPR)-like domains near the N-terminus, again possibly reflecting a specific PDK1 modification in the ancestor of these organisms. In summary, PDK1s from all 6 photosynthetic RAS species investigated lack lipid-binding domains, whereas some PDK1s from non-photosynthetic species have a lipid-binding domain and others do not.

More PDK1 sequences, particularly from the taxa underrepresented in Figure 28, must be identified before further investigating the predicted lipid-binding trends observed here to make sense of groups without a clear trend. It will also be interesting to test lipid binding specificity of diverse PDK1s and discover whether different clades regulate PDK1 via different lipid interactions, as animals, vascular plants, and at least some fungi seem to do.

4.5 PDK1 evolution

Perhaps because the majority of experimentally verified PDK1 homologues (Alessi et al., 1997b; Dong et al., 1999; Paradis et al., 1999; Casamayor et al., 1999; Niederberger and Schweingruber, 1999; Deak et al., 1999; Silber et al., 2004; Devarenne et al., 2006; Kamimura and Devreotes, 2010; Matsui et al., 2010; Dittrich and Devarenne, 2012) are relatively similar to each other in protein domain organization and

broad functional conservation, few research reports discuss PDK1 in an evolutionary context. After finding unexpected diversity in nonvascular plant and algae PDK1s, I hoped to learn more about the features of distantly related PDK1 and gain some insight into how these PDK1s could have arisen.

4.5a Limitations in discussing PDK1 evolution

Neither the phylogenetic grouping of eukaryotic speicies nor the root of the eukaryotic tree of life have been fully resolved (Tekle et al, 2009). While the Opisthokonta and Amoebozoa are well suported as monophyletic groups (Baldauf, 2008), Archaeplastida is controversial (Parfrey et al., 2010), and the placement of some species within the RAS and CCTH groups has changed in recent years (Parfrey et al., 2010; Baldauf, 2008; Dorrell and Smith, 2011; Tekle et al., 2009). Thus, the ancestral origins of current PDK1 sequences can be difficult to distinguish. Species thought to be early-diverging representatives of each eukaryotic group should be aggressively investigated and sequenced to illuminate our understanding of possible ancestral species and the evolution of PDK1 sequences.

4.5b PDK1 lethality

One aspect of PDK1 function that seems to differ between organisms is whether PDK1 is an essential gene. In animals and fungi, PDK1 null alleles have thus far proven to be lethal (Paradis et al., 1999; Casamayor et al., 1999; Niederberger and Schweingruber et al, 1999; Rintelen et al., 2001; Lawlor et al., 2002). In contrast, loss of PDK1 in Amoebozoa (Kamimura and Devreotes, 2010) and plants (Camehl et al., 2011; Dittrich and Devarenne, 2012) produces viable, though compromised, organisms. In

both *A. thaliana* and *P. patens*, all apparent PDK1 homologues (2 and 1 respectively) were knocked out without lethality (Camehl et al., 2011; Dittrich and Devarenne, 2012). Though these reports did not exclude the possibility that highly divergent PDK1 sequences are able to perform the same functions as the PDK1s that were knocked out, this possibility seems unlikely. Presence or absence of a PH domian does not appear to correlate with whether any particular PDK1 is essential or not, so trends regarding PDK1 lethality are difficult to predict in the 100 species investigated here.

Regardless, it is puzzling that a gene retained since the emergence of the earliest eukaryotes seems to be non-essential in at least some cases. Despite the fact that PDK1s from distantly related organisms can perform the same basic cellular functions (Dittrich and Devarenne, 2012), perhaps there are subtle differences in downstream components of PDK1 pathways, such as substrate AGC kinases, that make the difference between organism survival and death in the absence of PDK1. In the future perhaps PDK1 can be knocked out in genetically tractable organisms from different clades to better understand the circumstances that may enable PDK1 to become a non-essential gene.

4.5c PDK1 protein sizes and domains

The only evident trends in protein size and domain structure within the 100 putative PDK1s are those regarding presence or absence of a PH-like domain (Fig. 28; Fig. 29). Both the smallest PDK1s (with fewer than ~350 amino acids) and the largest (with more than 800 amino acids) are present in diverse groups of organisms, as are "typical" PDK1s of intermediate size with ~500-600 amino acids with a C-terminal PH domain (Fig. 28). This diversity complicates attempts to form a picture of PDK1

evolution, but might reflect rapid evolution in some lineages due to selective pressure, perhaps resulting in an unexpectedly wide variety of PDK1 activities. Very few large PDK1s contain conserved non-catalytic sequences identifiable by CDD search (Figure 28), so it is difficult to discuss the possible origins and functions of the large regions of sequence outside the kinase domain. One possible approach to study the evolutionary and functional implications of these regions is to perform deletion and "domain"-swapping experiments, followed by biochemical analysis of lipid binding and substrate phosphorylation in mutant proteins. Alternatively, it might be possible to simply perform a more sophisticated sequence analysis and arrive at possible functions of these large regions of sequence. However, the functions of the largest PDK1s cannot be effectively addressed without extensive sequence and/or functional analyses, so for the remainder of this section I focus on the evolution of PH-like domains within PDK1.

Animals and fungi are much closer relatives than animals and vascular plants (Baldauf, 2008), so it seems counterintuitive that animal and vascular plant PDK1s typically possess obvious PH-like domains, whereas only 3 of 21 fungal species investigated (*S. pombe*, *M. circinelloides*, and *G. intraradices*) do. Did PDK1 bind lipids in the ancestor of animals and fungi? One possibility is that PDK1s with PH domains arose separately in animals and fungi through convergent evolution. This explanation is reasonable, as PH domain promiscuity is well established in eukaryotes (Basu et al., 2008) and protein domain fusion is thought to occur more often than fission (Kummerfeld and Teichmann, 2005). However, the 3 fungal PDK1s with PH-like domains are not from close relatives in a monophyletic group (Grigoriev et al., 2012), so

these PDK1 PH domains must have been either added on several independent occasions or lost from multiple intervening lineages. Furthermore, 4 additional fungi (*M. globosa*, *P. graminis*, *S. complicata*, and *U. maydis*) belonging to several different clades (Grigoriev et al., 2012) have putative PDK1s with very weak PH-like regions on the C-terminal side of the kinase domain. Thus, another possibility is that these regions of weak similarity to PH domains might be due to incomplete nonfunctionalization; at some point the PDK1s had more recognizable PH domains, but a lack of selective pressure neither maintained nor got rid of them completely. For these reasons it may be that PDK1 in the Opisthokont ancestor have had a PH domain that has been retained in almost all animals, but is in various stages of being lost in many lineages of fungi for unknown reasons (Fig. 28; Fig. 29). Obtaining PDK1 sequences from more early-diverging Opisthokont species and performing lipid-binding tests with these PDK1s could be the first steps toward addressing these possibilities.

As mentioned previously, all vascular plants we investigated have putative PDK1s with a PH-like domain and are more similar to each other than PDK1s from *P. patens* and several species of algae, which is not particularly surprising since vascular plants are relatively recent innovations (Yoon et al., 2004). Similar to the fungi, putative PDK1s from nonvascular members of Archaeplastida vary substantially in size, with proteins ranging from ~300-800 amino acids (Fig. 28; Fig. 29). However, none of these sequences contain a detectable lipid-binding domain. Before asserting that this trend is a general one, it will be particularly important to obtain PDK1 sequences from glaucophyte and charophyte algae, as well as more species of red algae and nonvascular

land plants, such as liverworts and hornworts. If Archaeplastida are indeed monophyletic (Baldauf, 2008) and it remains true that the only representatives of Archaeplastida with PH-containing PDK1s are vascular plants, one possible explanation is that PDK1 in the Archaeplastidal ancestor lacked a PH domain, and then PDK1 in the ancestor of vascular plants acquired one from another gene. This explanation seems less plausible if in the future PDK1s with a PH domain, or with regions of weak similarity to a PH-like domain (like those in several fungi), are identified in many distantly related species of algae. In any case, it is intriguing that animal and vascular plant PDK1s are so similar in protein size, domain organization, and lipid binding capabilities given their distant evolutionary relationship and the diversity of PDK1 sequences found in closer relatives of both clades. In the future it will be interesting to assess whether this remarkable similarity may have arisen through convergent evolution due to selective pressure to efficiently regulate PDK1 or through some other mechanism, for example by many independent events of PH domain loss or nonfunctionalization.

4.5d Possible nature of PDK1 in the eukaryotic ancestor

Speculating about the nature of ancestral PDK1 sequences becomes increasingly difficult with more distantly related species. No groups of organisms (Amoebozoa, Opisthokonta, Excavata, Archaeplastida, or RAS) have PDK1s that universally lack a lipid-binding domain; PDK1s from Amoebozoa and Excavata all have a lipid-binding domain, but since only 7 representatives from these groups were investigated, this trend may not remain.

Recently the kinomes of 3 Excavates were analyzed: two amitochondriate species (the Diplomonad *Giardia lamblia* and the Parabasalid *Trichomonas vaginalis*) and one mitochondriate species (the Euglenozoan *Leishmania major*) (Manning et al., 2011). A single putative PDK1 was found in *G. lamblia*; it is was not used in my analysis because its annotation is ambiguous (Manning et al., 2011). In contrast, draft kinomes from *T. vaginalis* and *L. major* revealed 5 and 3 putative PDK1s respectively, and their sequences vary widely (Manning et al., 2011). A CDD search of all 8 *L. major* and *T. vaginalis* putative PDK1s detected a FYVE domain only in the putative *L. major* PDK1 used in this analysis and a PH-like domain only in the putative *T. vaginalis* PDK1 used in this analysis. Of course, it remains to be seen how many of the putative PDK1s in *L. major* and *T. vaginalis* are true PDK1 homologues. If the kinomes of *L. major* and *T. vaginalis* indeed contain several highly divergent PDK1 sequences, this suggests it is not beyond the realm of possibility for the ancestor of all eukaryotes to have had more than 1 PDK1-like gene in its genome. Ancient eukaryotes that were able to choose from several diverse PDK1-like proteins might have had more functional and regulatory possibilities, and thus a greater chance of surviving in different environments. It would be intriguing if the diversity of eukaryotic PDK1s could be at least partially explained by many instances of gene duplication and loss. In that case, it might be possible to find species whose genomes still contain multiple PDK1s with widely varying sizes and lipid-binding capabilities. Perhaps the *L. major* and *T. vaginalis* kinomes are just two examples of a phenomenon that was relatively common in the distant past, and might still be a feature of kinomes in some extant lineages.

4.6 Discussion

4.6a Discussion of plant and algae PDK1s

The initial phylogenetic analysis shows that there is a clear difference between higher (vascular) and lower (non-vascular) plants; the PDK1 proteins from these classes of plants can be distinguished by the presence or absence of the PH domain, respectively (Fig. 26). Thus, it is possible that the PH domain for lipid binding is an aspect of PDK1 function that developed at the time of vascular system formation. Additionally, the sequences for the green algae *O. lucimarinus* and *O. tauri*, the red alga *C. merolae*, and the brown alga *E. siliculosus* contain extensive amino acid sequences on the N-terminal and C-terminal sides of the kinase domain (Fig. 26; Fig. 28). The significance of these extra sequences towards function remains to be determined since activity of these proteins has not been reported. This analysis of plant PDK1 proteins is limited by the number of *PDK1* sequences from lower plants and non-flowering vascular plants. With the addition of genome sequences from additional lower plants such as the bryophyte liverwort *Marchantia polymorpha* (currently, the *M. polymorpha* EST sequences contain >4,000 contigs but not an obvious *PDK1* sequence homologue, http://www.genome.jp/kegg-bin/show_organism?org=empm), different species of algae (including charophyte and glaucophyte species), and vascular plants such as ferns, this comparison will become more robust and allow for a more concrete assessment of the domain evolution of PDK1 proteins.

4.6b Discussion of PDK1s from 100 diverse eukaryotes

The 100 putative PDK1s identified from more distantly related eukaryotes are more varied (Fig. 28; Fig. 29) than one might expect to find based on the small number of experimentally verified PDK1s, many of which are quite similar to each other. The diversity of putative PDK1s suggests that despite having a relatively conserved catalytic domain, PDK1 has nevertheless taken a number of different evolutionary paths since it first appeared, presumably in the ancestor of eukaryotes. The possible reasons for, and implications of, differences in PDK1 protein structure are still unknown, and in the future it will be interesting to test the degree of functional conservation amongst the most divergent PDK1 sequences. My analysis suggests that at least some PDK1 proteins may not be regulated by lipid interactions. If this is the case, a future goal should be to elucidate novel PDK1 regulatory mechanisms. Perhaps unidentified protein-interaction domains, other functional domains, or tight control of PDK1 substrate availability have facilitated PDK1 diversification in many species. Finally, once eukaryotic evolutionary relationships have been determined with more certainty, PDK1 sequences from organisms close to major divergence points should be investigated to uncover more clues about the nature of ancestral PDK1s in each eukaryote group.

CHAPTER V

CHARACTERIZATION OF A SECOND POTENTIAL PDK1 PHOSPHORYLATION SITE ON ADI3

5.1 Rationale

A previous phosphoamino acid analysis of PDK1-phosphorylated Adi3 suggested that Ser539 may not be the only residue at which Adi3 is phosphorylated by PDK1 (Devarenne et al., 2006). This is unexpected and interesting because currently no PDK1 phosphorylation events have been reported outside of the activation loop. Therefore, I further investigated the possibility of a second PDK1 phosphorylation site on Adi3.

5.2 Identification of a second putative PDK1 phosphorylation site on Adi3

5.2a Mass spectrometry-based search for Adi3 phosphorylation sites

The first approach to identify an additional PDK1 phosphorylation site relied on mass spectrometric analysis of kinase-inactive MBP-Adi3^{K337Q} that had been phosphorylated by MBP-PDK1 in vitro. A collaborator subjected PDK1-phosphorylated MBP-Adi3^{K337Q} to trypsin digest followed by tandem mass spectrometry to identify phosphorylated Adi3 peptides. This analysis identified 19 tryptic peptides of Adi3 (covering approximately 48% of Adi3), 4 of which contained a putative PDK1 phosphorylation site (Fig. 30). The putative PDK1 phosphorylation sites are Ser119, Ser518, Ser539, and either Thr679 or Ser680. The amino acid Ser651 had been identified as a putative PDK1 phosphorylation site in an earlier mass spectrometric analysis, but the peptide containing Ser651 was not identified in this analysis (Fig. 30).

Adi3:

MERIPEVRESTRQFPIGAKVAHTFSTSKKEVGIRGFRDFDLAIPIQTWKG
KTSYQEEEDLMVDAGTIKRSDDSLEDSGSTSFHGASHPPEPVDTDLMRPV
YVPIGQNKADGKCLVKNVSLKGPFLDDLSIRMPNVKPSPSLLSPAESLVE
EPNDLGVISSPFTVPRPSQNTETSLPPDSEEKECIWDASLPPSGNVSPLS
SIDSTGVVRSMSIVNSCTSTYRSDVLMSDGMLSVDRNYESTKGSIRGDSL
ESGKTSLSRASDSSGLSDDSNWSNITGSANKPHKGNDPRWKAILAIRARD
GILGMSHFKLLKRLGCGDIGSVYLSELSGTRCYFAMKVMDKASLASRKKL
TRAQTEREILQLLDHPFLPTLYTHFETDRFSCLVMEYCPGGDLHTLRQRQ
PGKHFSEYAARFYAAEVLLALEYLHMLGVVYRDLKPENVLVRDDGHIMLS
DFDLSLRCAVSPTLIRISSDDPSKRGAAFCVQPACIEPTTVCMQPACFLP
RLFPQKSKKKTPKPRADSGFQANSMPELVAEPTSARSMSFVGTHEYLAPE
IIKGEGHGSAVDWWTFGIFLHELLYGKTPFKGSGNRATLFNVVGQQLKFP
DSPATSYASRDLIRGLLVKEPQNRLGVKRGATEIKQHPFFEGVNWALIRC
STPPEVPRPVEPDYPAKYGQVNPVGVGNTSKRVVGADAKSGGKYLDFEFF

Figure 30. Summary of mass spectrometric identification of phosphorylation sites on Adi3. Diagram of Adi3 sequence, with tryptic peptides identified in the mass spectrometric analysis alternately highlighted in yellow and green. Putative PDK1 phosphorylation sites (S119, S518, S539, S651, and T679/S680) are indicated in red text, and putative Adi3 autophosphorylation sites (S256, S261) are indicated in blue text.

5.2b Testing Adi3 phosphorylation sites identified by mass spectrometry

Because previous experiments found that tomato PDK1 phosphorylates Ser rather than Thr residues (Devarenne et al., 2006), Ser119, Ser518, Ser651, and Ser680 were mutated to Ala in the MBP-Adi3^{K337Q} background, both individually and in various combinations with MBP-Adi3$^{K337Q/S539A}$. In vitro kinase assays were then performed to assess PDK1 phosphorylation of each protein (Fig. 31A). In accordance with previous results, phosphorylation of MBP-Adi3$^{K337Q/S539A}$ was reduced to about 60% of MBP-Adi3^{K337Q} (Devarenne et al., 2006). However, none of the other single Ser to Ala mutations were able to substantially reduce PDK1 phosphorylation of Adi3 (Fig. 31A, lanes 2-6). All higher-order Ser to Ala mutants (proteins with mutations at more than one site) tested displayed variable levels of phosphorylation by PDK1, but no single phosphorylation site (except Ser539) appeared to be consistently phosphorylated by PDK1 (Fig. 31A, lanes 7-14).

The next objective was to test whether phosphorylation of any of these residues activated Adi3, as Ser539 is known to do (Devarenne et al., 2006). This was accomplished by mutating Ser119, Ser518, Ser651, and Ser680 to the phosphomimetic residue Asp, both individually and in various combinations with MBP-Adi3^{S539D}. In vitro kinase assays were then performed to assess Adi3 autophosphorylation and its ability to phosphorylate the artificial kinase substrate myelin basic protein (Fig. 31B). Again in accordance with previous results, MBP-Adi3^{S539D} activity was increased approximately 4 fold compared to wild type MBP-Adi3 (Devarenne et al., 2006). However, none of the other Ser to Asp mutants were capable of substantially increased

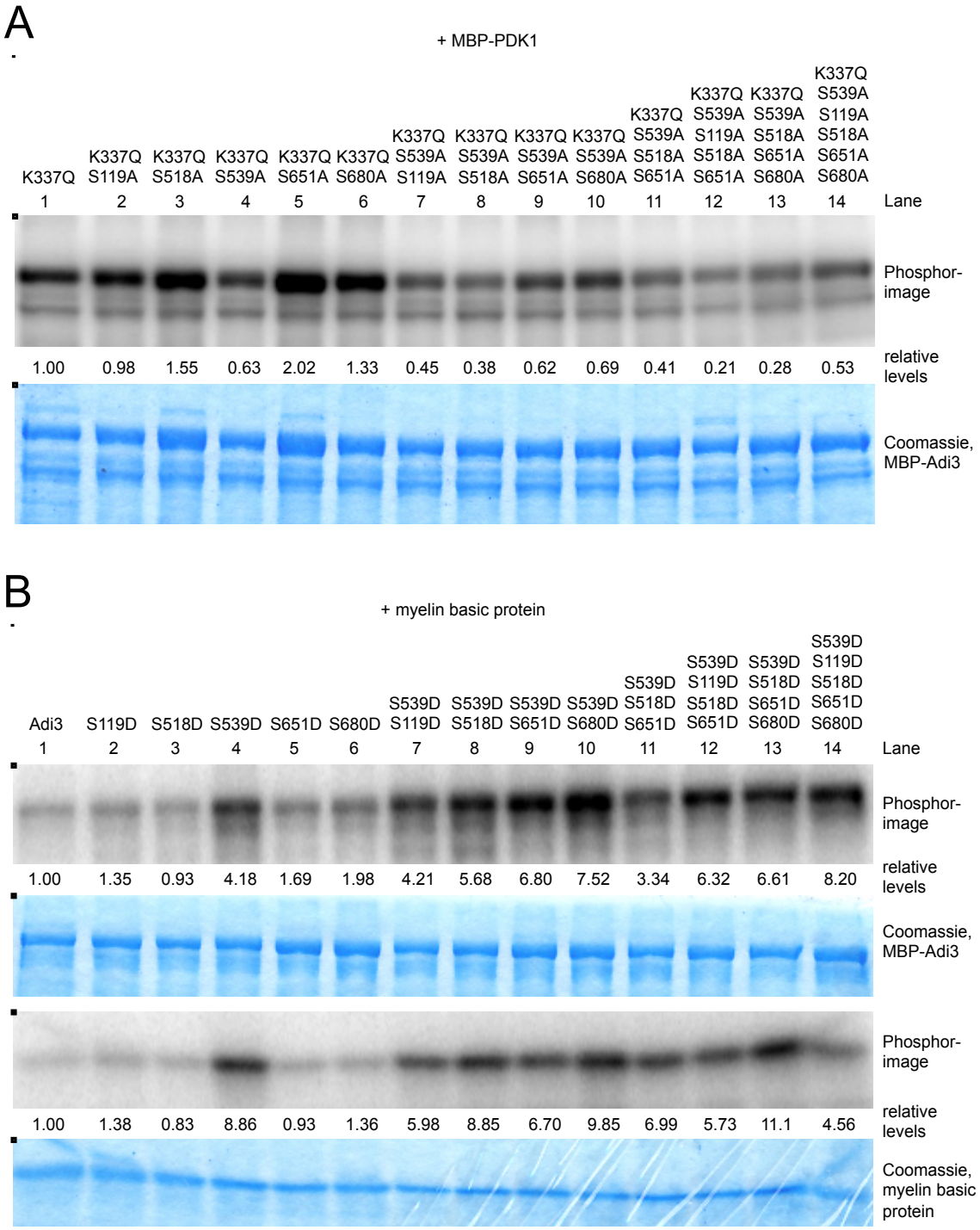
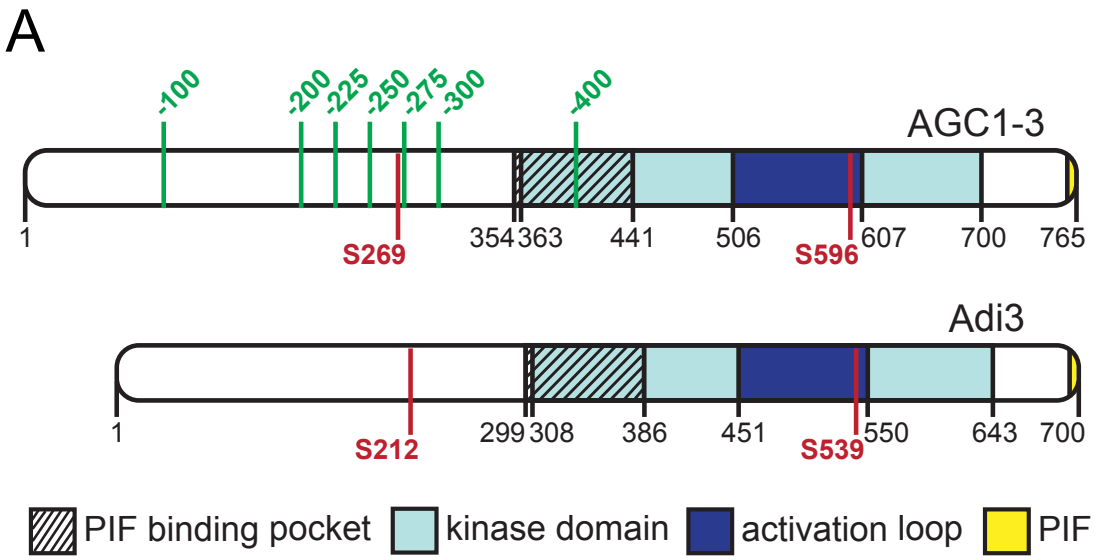


Figure 31. Investigation of phosphorylation sites on Adi3 identified by mass spectrometry. A, In vitro kinase assays to test PDK1 phosphorylation of different amino acids on Adi3. B, In vitro kinase assays to test activation of Adi3 by putative PDK1 phosphorylation sites. Adi3 autophosphorylation is shown in the upper phosphorimage, and Adi3 phosphorylation of myelin basic protein is shown in the lower phosphorimage.

autophosphorylation or phosphorylation of myelin basic protein (Fig. 31B, lanes 2-6). Higher-order Ser to Asp mutants displayed variable levels of autophosphorylation and phosphorylation of myelin basic protein, but no single phosphorylation site (except Ser539) was able to reproducibly activate Adi3 (Fig. 31B, lanes 7-14).

5.2c Identification of a second Adi3 phosphorylation site using N-terminal deletions

Because mass spectrometry was not able to identify any PDK1 phosphorylation sites on Adi3 that 1) were consistently phosphorylated by PDK1 and 2) reliably activated Adi3, an alternative approach was taken using the putative Adi3 homologue in *A. thaliana*, AGC1-3 (Gray and Devarenne, 2012). Successive N-terminal deletions of 100 and 25 amino acids were made in AGC1-3 (Gray and Devarenne, 2012; Fig. 32A), followed by phosphorylation with PDK1 to search for regions of AGC1-3 with substantially reduced PDK1 phosphorylation. This approach resulted in identification of a region between amino acids 250 and 275 of AGC1-3 that likely contained a PDK1 phosphorylation site (Gray and Devarenne, 2012; Fig. 32A). After comparing the serines in this region to the known PDK1 phosphorylation site on AGC1-3 (Ser596), it became apparent that the amino acids surrounding Ser269 share some similarities with those surrounding Ser596 (Fig. 32B), and thus both Ser269 and Ser539 might be phosphorylated by PDK1. Alignment of the AGC1-3 and Adi3 coding sequences revealed that Ser212 of Adi3 corresponds to Ser269 of AGC1-3. Unfortunately the tryptic peptide containing Ser212 was not identified in the previous attempt to find PDK1 phosphorylation sites using mass spectrometry (Fig. 30). As with AGC1-3, the



B

AGC1-3:	590	PN-TRSMSFVGTHEY	603
	262	MNLARAMSIANSSSA	276
Adi3:	532	PTSARSMSFVGTHEY	546
	205	TGVVRSMSIVNSCTS	219
	112	KCLVKNVSLKGPFLD	126
	511	TPKPRADSGFQANSM	525
	644	NWALIRCSTPPEVPR	658
	673	PVGVGNTSKRVVGAD	687

Consensus: S/T-F-V-G-T-X-E/D-Y

P +1 +2 +3 +4 +5 +6 +7

Figure 32. Identification of a second putative PDK1 phosphorylation site on AGC1-3 and Adi3. A, Diagram of AGC1-3 protein. N-terminal deletion mutations made by Joel Gray are indicated with green lines and text. PDK1 phosphorylation sites on AGC1-3 are indicated with red lines and text. The corresponding phosphorylation sites on Adi3 are also indicated with red lines and text. B, Alignment of the amino acids from P-7 to P+7 surrounding S596 and S269 in AGC1-3 and S539, S212, S119, S518, S651, and S680 in Adi3, with the phosphorylation sites indicated in red text. Amino acids similar or identical to those found in the consensus PDK1 phosphorylation motif of *A. thaliana* AGCVIIIa kinases (Zegzouti et al., 2006b) are underlined.

region surrounding Adi3 Ser212 shares some similarities with the region surrounding Ser539 (Fig. 32B), and with the PDK1 phosphorylation consensus motif found in mammalian and *S. cerevisiae* PDK1 substrates. The consensus PDK1 motif in mammals and yeast is Ser/Thr-Phe-Cys-Gly-Thr-X-Asp/Glu-Tyr, where the underlined Ser or Thr is the PDK1 phosphorylation site and X represents any amino acid (Cheng et al., 1998; Belham et al., 1999; Roelants et al., 2004). In *A. thaliana* kinases from the AGCVIIIa family Val has been substituted for Cys, but the rest of the consensus motif is the same (Fig. 32B; Zegzouti et al., 2006). The region surrounding Adi3 S212 lacks an amino acid similar to Gly in the P+3 position, Glu at P+6, or Tyr at P+7. However, Ile in the P+1 position is bulky and hydrophobic like Phe, and Ser in the P+4 position is similar to Thr (Fig. 32B). The region surrounding Adi3 S119 also has a bulky hydrophobic residue at P+1, but regions surrounding S518, S651, and S680 do not (Fig. 32B). Therefore, I hypothesized that Ser212 might be a second PDK1 phosphorylation site.

5.3 Testing phosphorylation of Adi3 Ser212 by PDK1 in vitro

After identification of Adi3 Ser212 as a potential secondary site of PDK1 phosphorylation, it was mutated to Ala in the kinase-inactive MBP-Adi3^{K337Q} background (Adi3$^{K337Q/S212A}$), both individually and in combination with the S539A mutation (Adi3$^{K337Q/S539A}$ and Adi3$^{K337Q/S212A/S539A}$). As expected, none of the mutations made in the MBP-Adi3^{K337Q} background were capable of autophosphorylation (Fig. 33A). These proteins were then used to assess PDK1 phosphorylation of Adi3 by in vitro kinase assays. In accordance with previous results, phosphorylation of MBP-Adi3$^{K337Q/S539A}$ was reduced to about 50% of MBP-Adi3^{K337Q} (Devarenne et al., 2006;

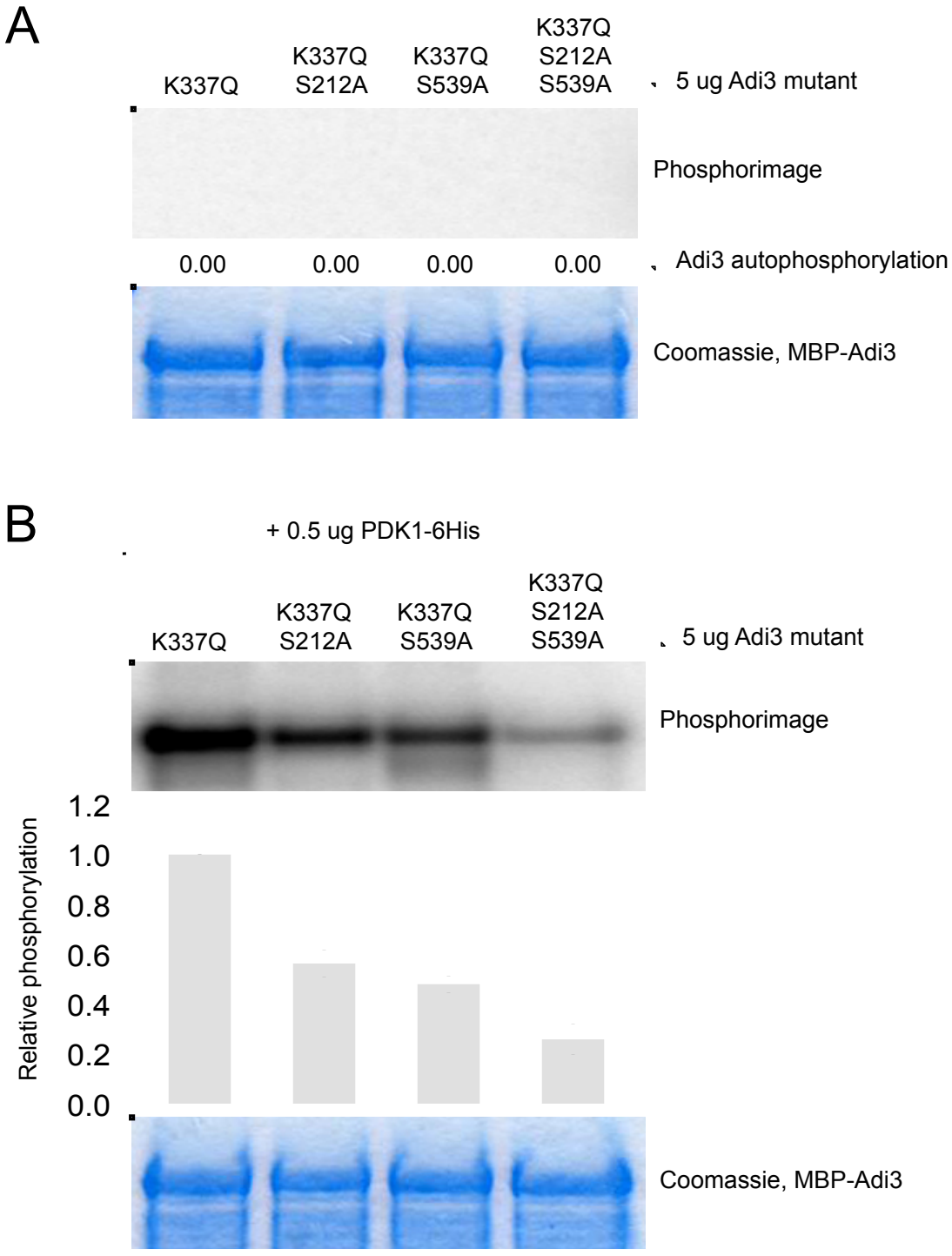


Figure 33. Phosphorylation of Adi3 Ser212 and Ser539 by PDK1. A, autophosphorylation of kinase inactive Adi3 mutant proteins. B, phosphorylation of kinase inactive Adi3 mutant proteins by PDK1. Error bars indicate standard error.

Fig. 33B). Similarly, phosphorylation of MBP-Adi3$^{K337Q/S212A}$ was reduced to about 60% of MBP-Adi3^{K337Q}, suggesting that PDK1 does phosphorylate Ser212 of Adi3. When both potential PDK1 phosphorylation sites were mutated to alanine in MBP-Adi3$^{K337Q/S212A/S539A}$, PDK1 phosphorylation was reduced to about 25% of MBP-Adi3^{K337Q}. Thus, there may be additional PDK1 phosphorylation sites on Adi3 that remain to be identified, but the similarity between Ser212, Ser539, and the PDK1 consensus motif suggests that Ser212 (Fig. 32B) may be the best candidate for future investigations. Furthermore, because previous work has found that two Adi3 peptides contain major sites of PDK1 phosphorylation but numerous peptides are phosphorylated to a much lesser extent (Devarenne et al., 2006), residual γ-[^{32}P] detected in the MBP-Adi3$^{K337Q/S212A/S539A}$ mutant might be due to low-level nonspecific PDK1 phosphorylation of many residues, which probably is not biologically significant.

5.4 Phosphorylation of Ser212 increases Adi3 activity on a substrate in vitro

Because none of the putative PDK1 phosphorylation sites identified by mass spectrometry were able to consistently activate Adi3 (Fig. 31B), it was important to test whether S212 phosphorylation increased Adi3 autophosphorylation or phosphorylation of a substrate. This was accomplished by mutating Ser212 to the phosphomimetic residue Asp, both individually (Adi3^{S212D}) and in combination with MBP-Adi3^{S539D} (Adi3$^{S212D/S539D}$). At the time of testing the S119, S518, S651, and S680 putative phosphorylation sites, no substrates of Adi3 had been identified. Since then Gal83, the β-subunit of the tomato SnRK1 complex, has been shown to be phosphorylated by Adi3 at

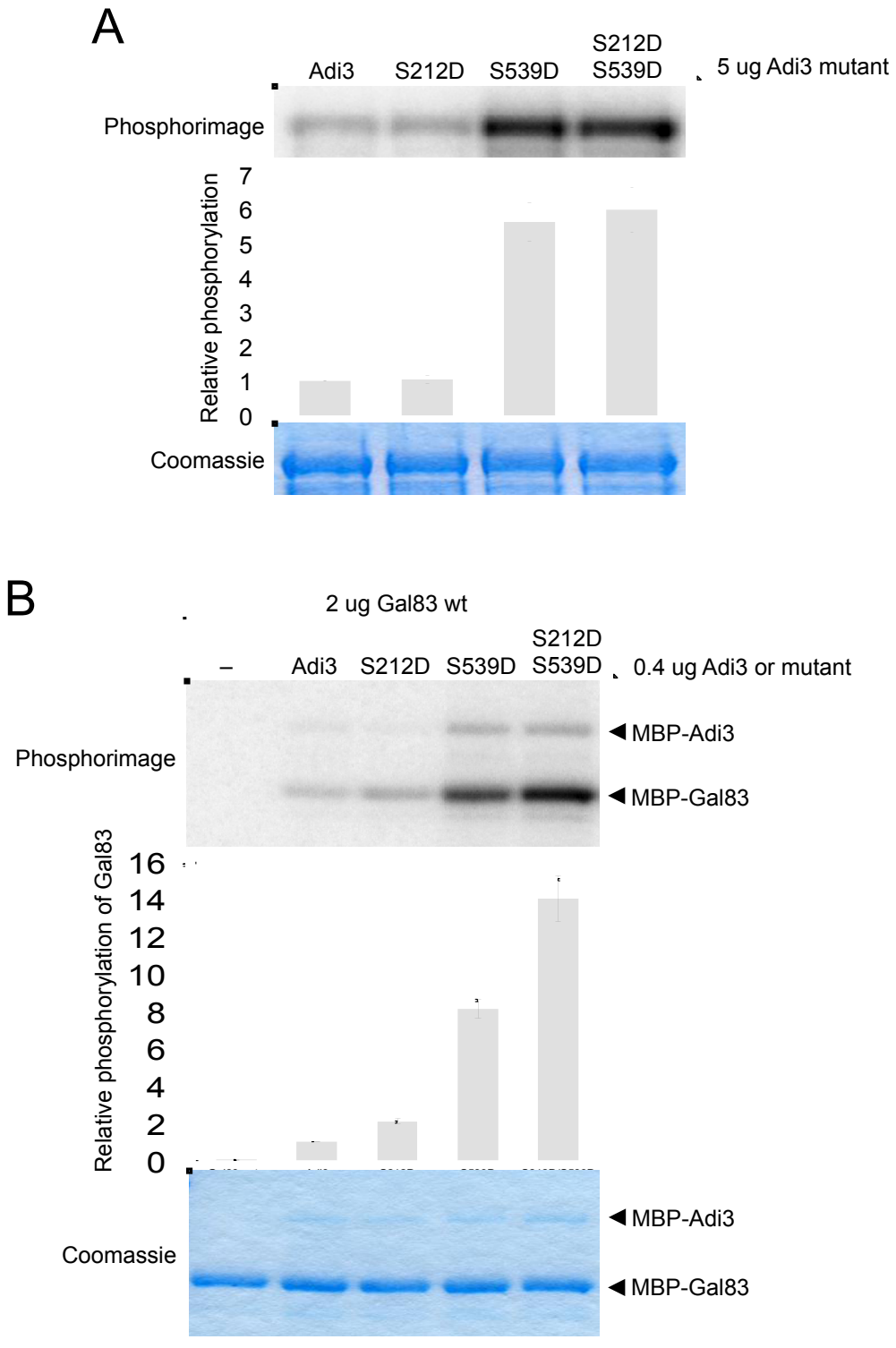


Figure 34. Phosphorylation of Ser212 only alters Adi3 activity on a substrate. A, Mutating S212 to the phosphomimetic amino acid aspartate does not increase Adi3 autophosphorylation, whereas mutating S539 to aspartate does increase Adi3 autophosphorylation. B, Mutating S212 to the phosphomimetic amino acid aspartate increases Adi3 phosphorylatioin of Gal83, though to a lesser extent than mutating S539 to aspartate. Error bars indicate standard error.

Ser26 (Avila et al., 2012). Thus, in vitro kinase assays were performed to assess Adi3 autophosphorylation and phosphorylation of Gal83.

5.4a Testing contribution of Ser212 to Adi3 activity

Unexpectedly, MBP-Adi3^{S212D} did not display increased autophosphorylation compared to MBP-Adi3, nor did MBP-Adi3$^{S212D/S539D}$ display increased autophosphorylation compared to MBP-Adi3^{S539D} (Fig. 34A). However, MBP-Adi3^{S212D} phosphorylation of Gal83 was increased approximately 2 fold compared to MBP-Adi3, and MBP-Adi3$^{S212D/S539D}$ phosphorylation of Gal83 was increased almost 2 fold compared to MBP-Adi3^{S539D} (Fig. 34B). While this activation of Adi3^{S212D} activity on a substrate is quite modest compared to Adi3^{S539D} activation, it has been consistently observed in multiple independent kinase assays, and is in contrast to the lack of reproducible activation by Adi3^{S119D}, Adi3^{S518D}, Adi3^{S651D}, or Adi3^{S680D}.

5.4b Comparing Adi3 phosphomimetic mutants with PDK1-phosphorylated Adi3

To further test whether PDK1 phosphorylation of Ser212 might contribute to Adi3 activation, a second in vitro kinase assay was performed to assess the degree to which PDK1 phosphorylation stimulates Adi3 activity on Gal83. In this experiment PDK1, Adi3, and Gal83 were mixed together to enable PDK1 to phosphorylate Adi3, and then Adi3 to phosphorylate Gal83. As a negative control PDK1 and Gal83 were mixed in the absence of Adi3 to ensure PDK1 did not phosphorylate Gal83. The extent of Gal83 phosphorylation in each case was compared with Gal83 phosphorylation by MBP-Adi3, Adi3^{S212D}, MBP-Adi3^{S539D}, and MBP-Adi3$^{S212D/S539D}$. PDK1 phosphorylation stimulated MBP-Adi3 activity on Gal83 by approximately 13 fold, comparable to the activity of

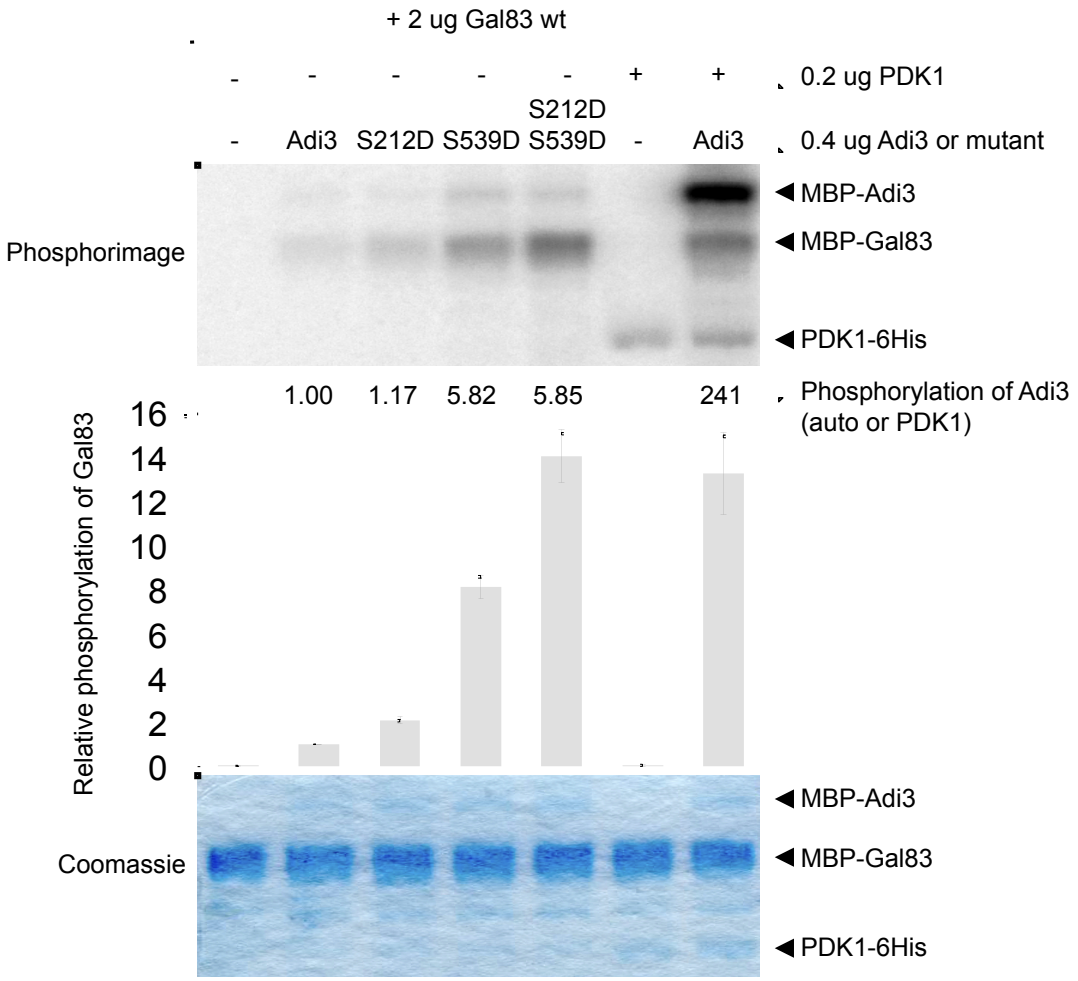


Figure 35. Activation of Adi3 through Ser212 and Ser539 phosphorylation accounts for all of Adi3's activity. Mutating either S212 or S539 to the phosphomimetic amino acid aspartate increases Adi3 autophosphorylation of Gal83. The double aspartate mutant protein has similar activity on Gal83 as Adi3 that has been fully phosphorylated by PDK1. Error bars indicate standard error.

MBP-Adi3$^{S212D/S539D}$ on Gal83 (Fig. 35). This result suggests that phosphorylation of both Ser212 and Ser539 could account for all, or almost all, of the activation of Adi3 that occurs upon PDK1 phosphorylation.

5.5 Resolution of differentially phosphorylated forms of Adi3

The next step in investigating Ser212 as a potential PDK1 phosphorylation site on Adi3 is to confirm that PDK1 phosphorylates Ser212 in vivo. For initial experiments I hoped to use a simple method to obtain preliminary evidence regarding the phosphorylation state of Adi3. One method that has been reported to separate differentially phosphorylated proteins is to perform SDS-PAGE using protein gels with decreased ratios of the bisacrylamide crosslinker (Demmel et al., 2008).

Purified MBP-Adi3, Adi3$^{K337Q/S212A}$, MBP-Adi3$^{K337Q/S539A}$, and MBP-Adi3$^{K337Q/S212A/S539A}$ were phosphorylated by PDK1 and then separated by SDS-PAGE with a 200:1 ratio of acrylamide:bisacrylamide. When PDK1 was not added to the phosphorylation reaction, all MBP-Adi3 proteins migrated as a single band (Fig. 36), in accordance with the fact that the K337Q mutation renders them incapable of autophosphorylation (Devarenne et al., 2006). However, addition of PDK1 to MBP-Adi3 resulted in the appearance of two phosphorylated forms of the protein that migrated more slowly than unphosphorylated MBP-Adi3 (Fig. 36). Both Adi3$^{K337Q/S212A}$ and MBP-Adi3$^{K337Q/S539A}$ displayed only two bands upon PDK1 phosphorylation, and Adi3$^{K337Q/S212A/S539A}$ migrated as a single band, similar to unphosphorylated MBP-Adi3.This result suggests that SDS-PAGE with reduced ratios of bisacrylamide may be a promising technique for resolving differentially phosphorylated species of Adi3

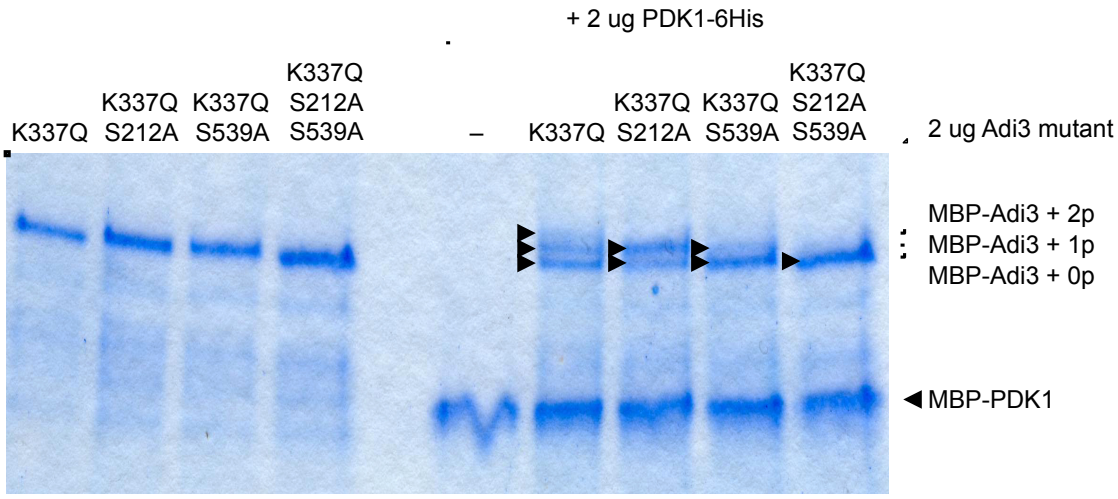


Figure 36. Separation of PDK1-phosphorylated Adi3 using gels with low ratios of crosslinker. Differentially phosphorylated species of Adi3 can be resolved from each other when SDS-PAGE is performed using gels in which the ratio of acrylamide to bisacrylamide is reduced from 29:1 or 37.5:1 to 200:1. Different species of Adi3 are indicated by arrowheads next to the band.

expressed in plant tissue, such as protoplasts. Interestingly, the relative abundances of each MBP-Adi3 species appear to differ (Fig. 36), raising the possibility that in vitro Ser539 is phosphorylated by PDK1 to a greater extent than Ser212. Future experiments should investigate this possibility further, especially for Adi3 expressed in plant tissue.

One approach I have considered for future experiments is to overexpress PDK1 and GFP-Adi3 in tomato or *A. thaliana* protoplasts, then immunoprecipitate Adi3 with a GFP antibody and identify phosphorylation sites by mass spectrometry. Indeed, mass spectrometric validation of Ser212 as a true PDK1 phosphorylation site will be critical to perform before this work may be published, there are several difficulties that should be considered. First, protoplasts can express relatively high levels of proteins when transiently transfected with a gene whose expression is driven by a strong promoter (e.g. the Cauliflower Mosaic Virus 35S promoter), but low yields of protoplasts limit the amount of protein that may be obtained. Second, immunoprecipitation requires several hours to perform, and Adi3 has been observed to be degraded rather rapidly in cell lysates. Finally, previous attempts to perform mass spectrometry on approximately 25 μg of purified MBP-Adi3 led to identification of peptides covering only 48% of the protein (Fig. 30). Thus, optimizing a protocol that will enable mass spectrometric identification of peptides covering all of Adi3 may take some time.

5.6 Discussion

The experiments presented in this chapter provide preliminary evidence that Adi3 may possess a second PDK1 phosphorylation site at Ser212, which is distinct from the highly conserved activation loop site at Ser539 but shares some similarity in

neighboring amino acids thought to direct PDK1 phosphorylation (Fig. 32B). Phosphorylation at multiple sites could provide an additional level of regulation to Adi3 interactions by activating Adi3 to different degrees (Fig. 34; Fig. 35), or possibly by altering other aspects of Adi3 function, such as subcellular localization or the ability to interact with non-substrate partners like 14-3-3 proteins (Johnson et al., 2010). However, it is important to note I have not ruled out the possibility that the PDK1 phosphorylation of Adi3 Ser212 presented here is simply an artifact of in vitro kinase assays. In order to confirm Ser212 as a biologically relevant phosphorylation site it is necessary to test whether Adi3 expressed in plant tissue is phosphorylated at this residue. Before optimizing an Adi3 expression and purification technique that will facilitate mass spectrometry to identify in vivo phosphorylation sites, SDS-PAGE with low amounts of bisacrylamide may be used to initially test the phosphorylation status of Ser212 in vivo.

If Ser212 is indeed phosphorylated in vivo, it will be interesting to investigate whether phosphorylation at multiple sites is a common feature of plant PDK1-substrate interactions. Mutating the conserved activation loop site does not completely abolish in vitro PDK1 phosphorylation of several plant AGC kinases, including OXI1 in rice (Matsui et al., 2010), an uncharacterized AGC kinase in moss (Dittrich and Devarenne, 2012), and AGC1-3 in *A. thaliana* (Gray and Devarenne, 2012). In general, little is known about the possibility of multiple PDK1 phosphorylation events within a protein. If amino acids outside the activation loop are confirmed as true PDK1 phosphorylation sites, it will be important to establish a phosphorylation consensus motif for these sites, as has been reported for the activation loop site (Cheng et al., 1998; Belham et al., 1999;

Roelants et al., 2004; Zegzouti et al., 2006b). Interestingly, some AGC kinases like PINOID appear to be phosphorylated by PDK1 only in the activation loop (Zegzouti et al., 2006a), but have a serine that corresponds to S212 in Adi3 (data not shown). Others like KIPK appear not to be phosphorylated at all by PDK1, despite possessing a completely conserved activation loop phosphorylation motif (Zegzouti et al., 2006). Thus, it is apparent that our understanding of PDK1-substrate interactions is still far from complete.

This work does not address the possibility that Adi3 and other plant AGC kinases may possess additional regulatory phosphorylation sites unrelated to PDK1. In mammalian systems a conserved phosphorylation site has been identified in the "turn" motif of many AGC kinases, which is located near the C-terminus of the protein (Hauge et al., 2007). Phosphorylation of the turn motif appears to promote association of the PIF with its binding pocket, thereby favoring the activated conformation of the kinase. This turn motif site does not appear to be strictly conserved in Adi3 (the corresponding amino acid in Adi3 is a valine), though Thr679 and Ser680 are nearby and could theoretically perform a similar function. To test this possibility the activity of MBP-Adi3 should be compared with MBP-Adi3^{T679A} or MBP-Adi3^{S680A}, and also with MBP-Adi3^{T679D} or MBP-Adi3^{S680D}. If these sites are indeed phosphorylated in Adi3, PDK1 is unlikely to be the kinase that phosphorylates them (Hauge et al., 2007; Fig. 30A).

Another aspect of Adi3 function that has not yet been addressed is the contribution of autophosphorylation to protein stability or activity on substrates. Mass spectrometry previously identified 2 potential autophosphorylation sites at Ser256 and

Ser261 (Fig. 30), but these sites might not be relevant to Adi3 function, as seems to be the case with Ser119, Ser518, Ser651, and Ser680. The N-terminal deletion strategy employed to find PDK1 phosphorylation sites on AGC1-3 and Adi3 (Fig. 32A) is probably not an effective way to search for autophosphorylation sites because there are several reasons a deletion mutation might compromise kinase autophosphorylation. In the future, mass spectrometry should be repeated with autophosphorylated Adi3, and candidate sites of autophosphorylation should be tested using experiments similar to those discussed here.

CHAPTER VI

PRODUCTION OF ANAGOGUE-SENSITIVE ADI3 FOR SUBSTRATE SEARCH*

6.1 Rationale

Protein kinase substrates are sometimes difficult to identify using interaction-based methods like a yeast two-hybrid screen. A screen for Adi3 interacting proteins recovered many interactors, but only 1 evident substrate. Therefore, in an attempt to isolate additional Adi3 substrates, I developed an ATP analog-sensitive (*as*-) form of Adi3.

6.2 Identification of a target Adi3 gatekeeper residue

In this approach, an *as*-kinase is produced by mutating a bulky, or "gatekeeper", amino acid within the ATP-binding pocket to a less bulky amino acid such as Ala or Gly (Koch and Hauf, 2010; Dephoure et al., 2005). This creates a larger ATP-binding pocket and allows for the specific use of bulky forms of ATP with N6 substitutions such as N6-benzyl-ATP or N6-phenylethyl-ATP (Koch and Hauf, 2010; Dephoure et al., 2005; Fig. 37). Only the *as*-kinase can utilize the bulky ATP analogs and thus, can be used to specifically identify phosphorylation substrates from cell extracts. The production and use of *as*-kinases has been successfully used on dozens of kinases from yeast and mammalian systems (Koch and Hauf, 2010; Zhang et al., 2005), but this technology has rarely been used on plant kinases (Zhang et al., 2005; Bohmer and Romeis, 2007;

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*Portions of the following article have been reprinted with permission: **Dittrich ACN, Devarenne TP** (2012) An ATP analog-sensitive version of the tomato cell death suppressor protein kinase Adi3 for use in substrate identification. Biochimica et Biophysica Acta Proteins and Proteomics **1824:** 269-273. Copyright 2012 © Elsevier.

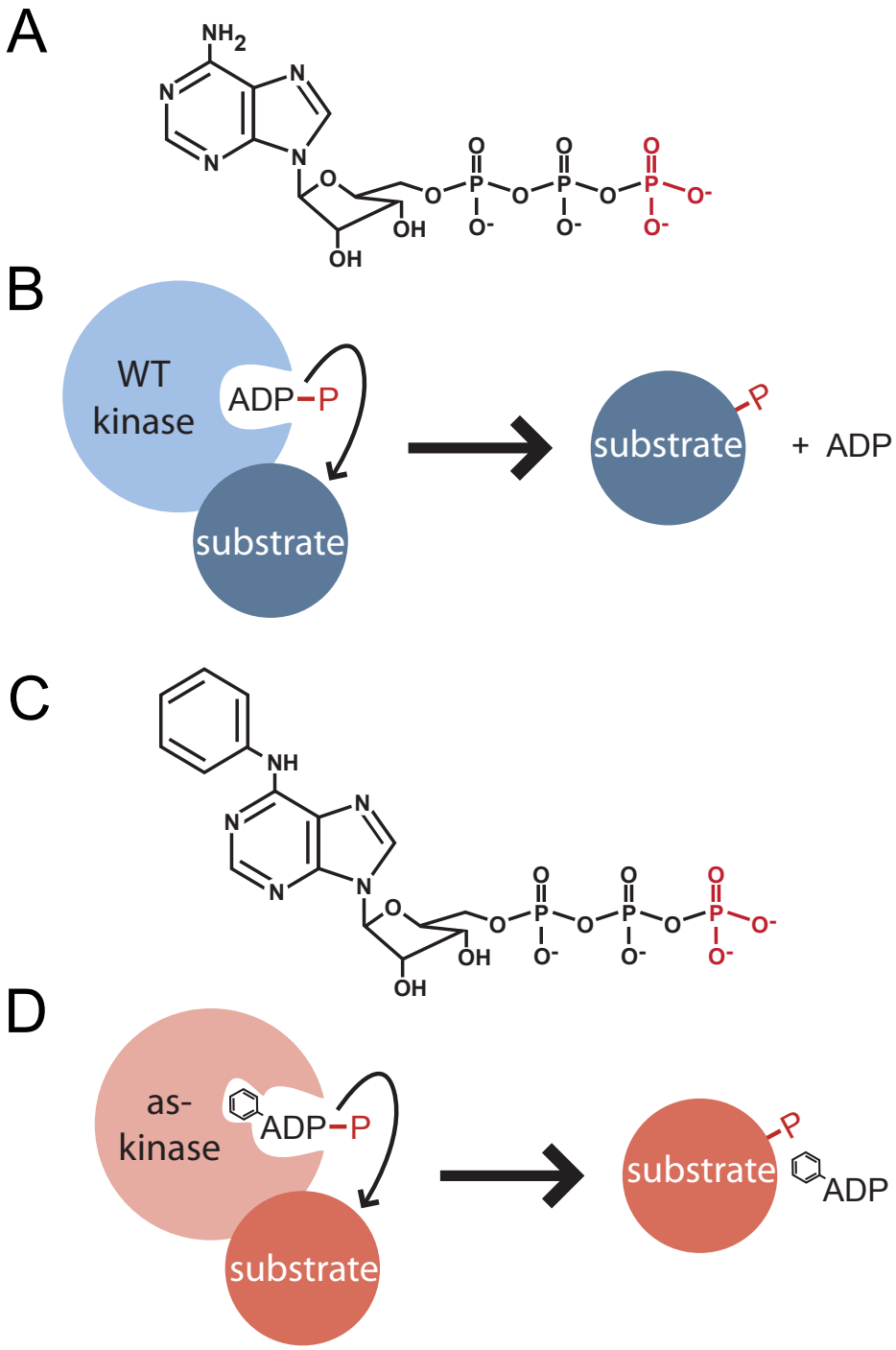

Fig. 37. Schematic of the analog-sensitive kinase technology. A, Structure of ATP with gamma phosphate shown in red. B, A wild-type protein kinase use of the γ- phosphate of ATP to phosphorylate substrates. C, Structure of N6-benzyl-ATP with gamma phosphate shown in red. D, An *as*-protein kinase use of the γ-phosphate of bulky ATP analogs to phosphorylate substrates.

Salomon et al., 2009a; Salomon et al., 2009b). Here I report the development of an *as*-form of Adi3 (*as*-Adi3) and show that it can specifically utilize N6-benzyl-ATP to phosphorylate a known Adi3 substrate.

The first step in the production an *as*-kinase is the identification of the gatekeeper residue that when mutated will allow for use of bulky ATP analogs. Isoleucine 338 of the Rous sarcoma virus tyrosine protein kinase v-Src has been identified as the gatekeeper amino acid (Shah et al., 1997). This residue was identified by analyzing the structures of two other kinases, PKA and CDK2, to locate the residue(s) closest to the N6- amine group of ATP; Met120 in PKA and Phe80 in CDK2 (Fig. 38A). Alignment of the v-Src protein sequence with that of PKA and CDK2 identified v-Src Ile338 as a potential mutational target and analysis confirmed that Ile338 mutation allowed for use of N6-benzyl-ATP (Shah et al., 1997). Thus, Adi3 was aligned with PKA, CDK2, and v-Src, and Met385 of Adi3 was identified as a potential mutational target (Fig. 38A).

6.3 Mutation of Adi3 Met385 to Ala or Gly enables use of N6-benzyl-ATP

Previous studies have shown that a fusion of Adi3 to MBP produces soluble, kinase-active protein that effectively mimics native activity (Devarenne et al., 2006; Ek-Ramos et al., 2010). Adi3 Lys337 was identified as the invariant Lys in the ATP-binding pocket that coordinates ATP binding, and Ser539 as the upstream activation site on Adi3 (Devarenne et al., 2006). Mutation of Adi3 Lys337 to Gln ($Adi3^{K337Q}$) produces an inactive protein kinase and mutation of Ser539 to Asp ($Adi3^{S539D}$) produces a constitutively active Adi3 (Devarenne et al., 2006). After identification of Adi3 Met385 as a potential gatekeeper amino acid it was mutated to Ala or Gly in the MPB-Adi3,

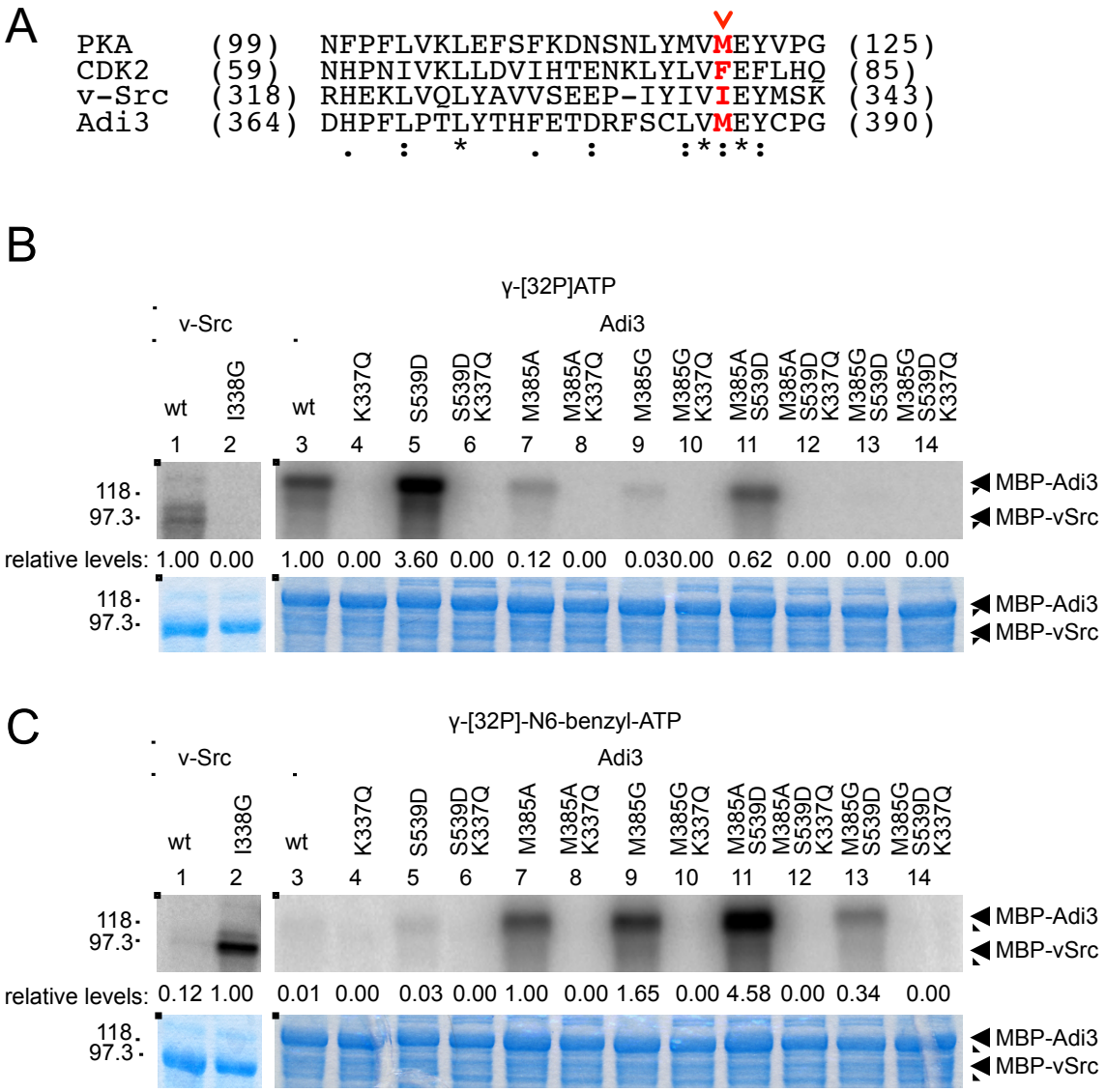

Fig. 38. Mutation of Adi3 Met385 allows use of bulky ATP analogs. A, Identification of the target Adi3 gatekeeper residue (in red text and indicated with arrow) by alignment with PKA, CDK2, and v-Src. In (B) and (C) the indicated proteins were incubated in an *in vitro* kinase assay separated by SDS-PAGE, and exposed to a phosphorimager. Top panel, phosphorimage; bottom panel, Coomassie stained gel. B, Adi3 M385A and M385G mutations limit the use of γ-[32P]ATP in autophosphorylation. C, Adi3 M385A and M385G mutations can use γ-[32P]-N6-benzyl- ATP in autophosphorylation.

MBP-Adi3^{K337Q}, and MPB-Adi3^{S539D} backgrounds.

First, the ability of the Adi3 *as*-mutants to utilize ATP for autophosphorylation was tested using *in vitro* kinase assays and γ-[^{32}P]ATP. An MBP tagged version of v-Src^{I338G} was used as a positive control for non-use of ATP. As has been previously reported (Shah et al., 1997), v-SrcI338G was not capable of utilizing ATP for autophosphorylation (Fig. 38B, lane 2). As reported previously (Devarenne et al., 2006), the Adi3^{K337Q} protein is autophosphorylation-inactive (Fig. 38B, lane 4) and Adi3^{S539D} had greatly increased autophosphorylation over that of wild-type Adi3 (Fig. 38B, compare lanes 3 and 5). The Adi3^{M385A} and Adi3^{M385G} proteins could both utilize ATP to a low level with Adi3^{M385A} being more efficient (Fig. 38B, lanes 7 and 9). Introduction of the M385A and M385G mutations into the Adi3^{S539D} background increased the ability of the Adi3^{M385A} protein to utilize ATP (Fig. 38B, lane 11), and actually decreased the ability of Adi3^{M385G} to use ATP (Fig. 38B, lane 13). The K337Q mutation in any combination with Adi3^{M385A} or Adi3^{M385G} eliminated the ability of Adi3 to utilize ATP (Fig. 38B, lanes 8, 10, 12, 14). These studies indicate that the Ala or Gly mutations of Met385 decrease, but do not fully eliminate, the ability of Adi3 to utilize ATP. However, the M385G mutation provides a near complete loss of ATP use especially in the Adi3^{S539D} background.

In order to test the ability of the *as*-Adi3 mutants to utilize N6-benzyl-ATP, radiolabeled γ-[^{32}P]-N6- benzyl-ATP was produced using NDPK and γ-[^{32}P]ATP to phosphorylate non-labeled N6-benzyl-ADP. As expected, v-Src^{I338G} efficiently utilized γ-[^{32}P]-N6-benzyl-ATP while the wild-type v-Src could not (Fig. 38C, lane 1 and 2).

Wild-type Adi3 and Adi3^{K337Q} could not utilize γ-[^{32}P]-N6-benzyl-ATP, while Adi3^{S539D} had a very low ability to use γ-[^{32}P]-N6-benzyl-ATP (Fig. 38C, lanes 3, 4, and 5). The M385A mutation in the wild-type Adi3 or Adi3^{S539D} backgrounds allowed for use of γ-[^{32}P]-N6-benzyl-ATP, with Adi3$^{M385A/\ S539D}$ having the expected increase in activity over Adi3^{M385A} (Fig. 38C, lanes 7 and 11). The M385G mutation in the wild-type Adi3 or Adi3^{S539D} backgrounds allowed for use of γ-[^{32}P]-N6-benzyl-ATP, however, Adi3$^{M385G/S539D}$ did not have the expected increase in activity as compared to Adi3^{M385G} (Fig. 38C, lanes 9 and 13). These results indicate that mutation of Adi3 Met385 to Ala or Gly enlarges the ATP binding pocket to allow for use of N6-benzyl-ATP. Since the Adi3^{M385G} protein utilizes ATP less than the Adi3M385A protein, especially in the activated background (Adi3$^{M385G/S539D}$; Fig. 38B), I performed follow-up studies on the Adi3^{M385G} and Adi3$^{M385G/S539D}$ proteins.

6.4 Elimination of background use of ATP

Since the Adi3^{M385G} and Adi3$^{M385G/S539D}$ proteins can utilize γ-[^{32}P]ATP to a low level (Fig. 38B), experiments were carried out to determine if use of γ-[^{32}P]ATP by the Adi3^{M385G} and Adi3$^{M385G/S539D}$ proteins can be blocked through the use of two bulky ATP analogs. For these assays, Adi3 protein was preincubated with non-radiolabeled N6-benzyl-ATP or N6-phenylethyl-ATP followed by incubation in a kinase assay with γ-[^{32}P]ATP. If the bulky ATP analogs are efficiently used by the Adi3 proteins, the incorporation of [^{32}P] from γ-[^{32}P]ATP should be eliminated. The results show that bulky ATP analog preincubation did not affect the ability of wild-type Adi3 or Adi3^{S539D} to incorporate [^{32}P] (Fig. 39, lanes 1, 3, 5, and 7), while the preincubation eliminated the

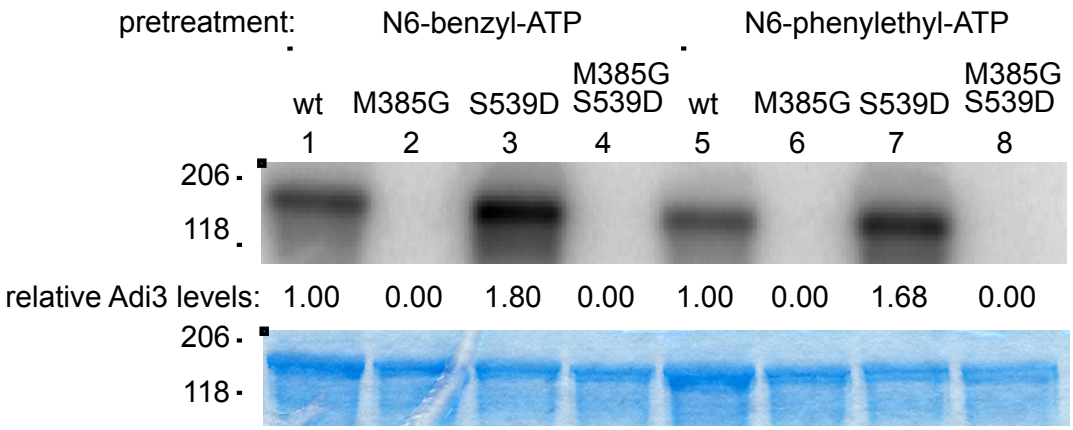

Fig. 39. Background use of ATP by Adi3M385G can be competed by bulky ATP analogs. The indicated MBP-Adi3 proteins were incubated with N6-benzyl-ATP or N6-phenylethyl-ATP prior to incubation with γ-[32P]-ATP. Samples were analyzed as in Fig. 2. Top panel, phosphorimage; bottom panel Coomassie stained gel.

background use of γ-[^{32}P]ATP by Adi3^{M385G} and Adi3M385G/S539D (Fig. 39, lanes 2, 4, 6, and 8). This would indicate that the background use of ATP by the *as*-Adi3 proteins can be eliminated in the presence of the bulky ATP analogs.

6.5 Adi3M385G/S539D can utilize N6-benzyl-ATP to phosphorylate a known substrate

Finally, I examined if the Adi3$^{M385G/S539D}$ protein could phosphorylate a known Adi3 substrate. Gal83, the β-subunit of the tomato SnRK1 protein complex, has been shown to be phosphorylated by Adi3 at Ser26 (Avila et al., 2012). The Adi3$^{M385G/S539D}$ protein was capable of utilizing γ-[^{32}P]-N6-benzyl-ATP to phosphorylate Gal83 and this phosphorylation was eliminated by the Gal83 Ser26A mutation (Fig. 40). Elimination of Adi3$^{M385G/S539D}$ kinase activity by introduction of the K337Q mutation resulted in a loss of Gal83 phosphorylation (Fig. 40).

6.6 Discussion

6.6a Potential future use of analogue-sensitive Adi3

These results indicate that mutation of the Adi3 Met385 residue produces an analog-sensitive version of Adi3 and enables the use of N6-benzyl-ATP for phosphotransfer in both autophosphorylation and *trans*-phosphorylation. This indicates that the *as*-Adi3 proteins could be used in the future for the identification of substrates from plant protein extracts. This could be accomplished through the direct phosphorylation of substrates with γ-[^{32}P]-N6-benzyl-ATP (Dephoure et al., 2005) or through inhibiting phosphorylation with γ-[^{32}P]ATP since *as*-kinases can also specifically bind ATP analogue inhibitors such as 1-NA-PP1 (Bohmer and Romeis,

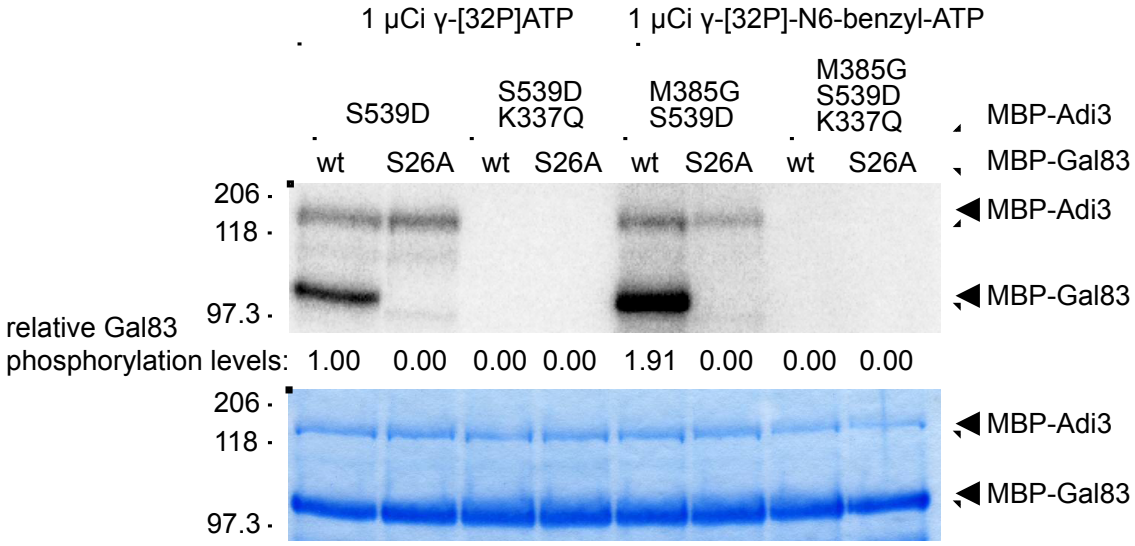

Fig. 40. Adi3M385G/S539D can use γ-[32P]-N6-benzyl-ATP to phosphorylate Gal83 in kinase assays. Samples were analyzed as in Fig. 2. Top panel, phosphorimage; bottom panel, Coomassie stained gel.

2007; Salomon et al., 2009a; Bishop et al., 2001). Stable Adi3 knockout lines are necessary for the second method, and these are not available in tomato, so direct phosphorylation seems to be the best approach. It is important to note that mutation of only Met385 was required for use of N6-benzyl-ATP by Adi3. This is of importance because in some kinases, mutation of the gatekeeper residue does not allow for use of bulky ATP analogs. These kinases require a second-site mutation to produce a fully active *as*-kinase (Zhang et al., 2005). In this sense, Adi3 behaves like the majority of other kinases for which *as*- versions have been made. These studies are also of significance since only a few plant protein kinases have been mutated to an *as*-kinase; the tomato protein kinases Pto and MPK3, the tobacco kinase CDPK2, and the *Arabidopsis* kinases CPK1 and MPK4 (Zhang et al., 2005; Salomon et al., 2009a; Salomon et al., 2009b; Bohmer and Romeis, 2007; Brodersen et al., 2006). Our studies indicate that it is possible to use this approach on more plant kinases.

6.6b Problems with using analogue-sensitive Adi3 to discover Adi3 substrates

Unfortunately, though bulky ATP analogs are now known to be compatible with the active sites of multiple plant kinases, it has proved challenging to move from in vitro work to the identification of direct kinase substrates in plant tissue extracts. In contrast to other organisms (Shah et al., 1997; Zhang et al., 2005; Ubersax et al., 2003), no published reports have identified plant kinase substrates by using gatekeeper mutations in combination with radiolabeled bulky ATP analogs like N6-benzyl-ATP. The only publication that has discovered novel plant kinase substrates via an *as*-kinase relied on transgenic *A. thaliana* lacking the wild type kinase and transformed with an *as*-kinase,

whose activity was then specifically suppressed with the inhibitor 1-NA-PP1. Two-dimensional electrophoresis of total plant proteins from wild type and transgenic plants enabled comparison of phosphorylated proteins in each extract, followed by subsequent identification of proteins that were not phosphorylated in the presence of the *as*-kinase inhibitor (Bohmer and Romeis, 2007). As mentioned above, this approach is currently not feasible for tomato because it requires stably transformed knockout lines.

6.6c Unsuccessful attempts to discover Adi3 substrates

I attempted to directly phosphorylate as-Adi3 substrates many times using a variety of techniques, including 1) isolating nuclei to enrich for potential Adi3 substrates (Ek-Ramos et al., 2010), 2) including phosphatase/ATPase inhibitors like sodium orthovanadate and sodium fluoride, 3) adding high concentrations (up to 2 mM) of unlabeled ATP to saturate endogenous proteins that might be able to use the radiolabeled analog, 4) using a phosphocreatine/creatine kinase ATP-regenerating system to keep ATP concentrations high during the course of the reaction (Ubersax et al., 2003; Fig. 41), 5) adding high concentrations (up to 2 mM) of the nonhydrolyzable ATP analog ATPγS to inhibit endogenous proteins that might degrade the radiolabeled analog (Fig. 42), and 6) desalting plant extract with Sephadex resin to remove small molecules that might adversely affect the reaction. None of these methods were successful; all incubation of *as*-Adi3 with plant extracts resulted in a decrease in *as*-Adi3 autophosphorylation, as well as varying degrees of nonspecific γ-[^{32}P] incorporation into numerous plant proteins. No specific phosphorylation of an endogenous substrate was detected in any of my experiments (Fig. 41; Fig. 42). This problem could be partially

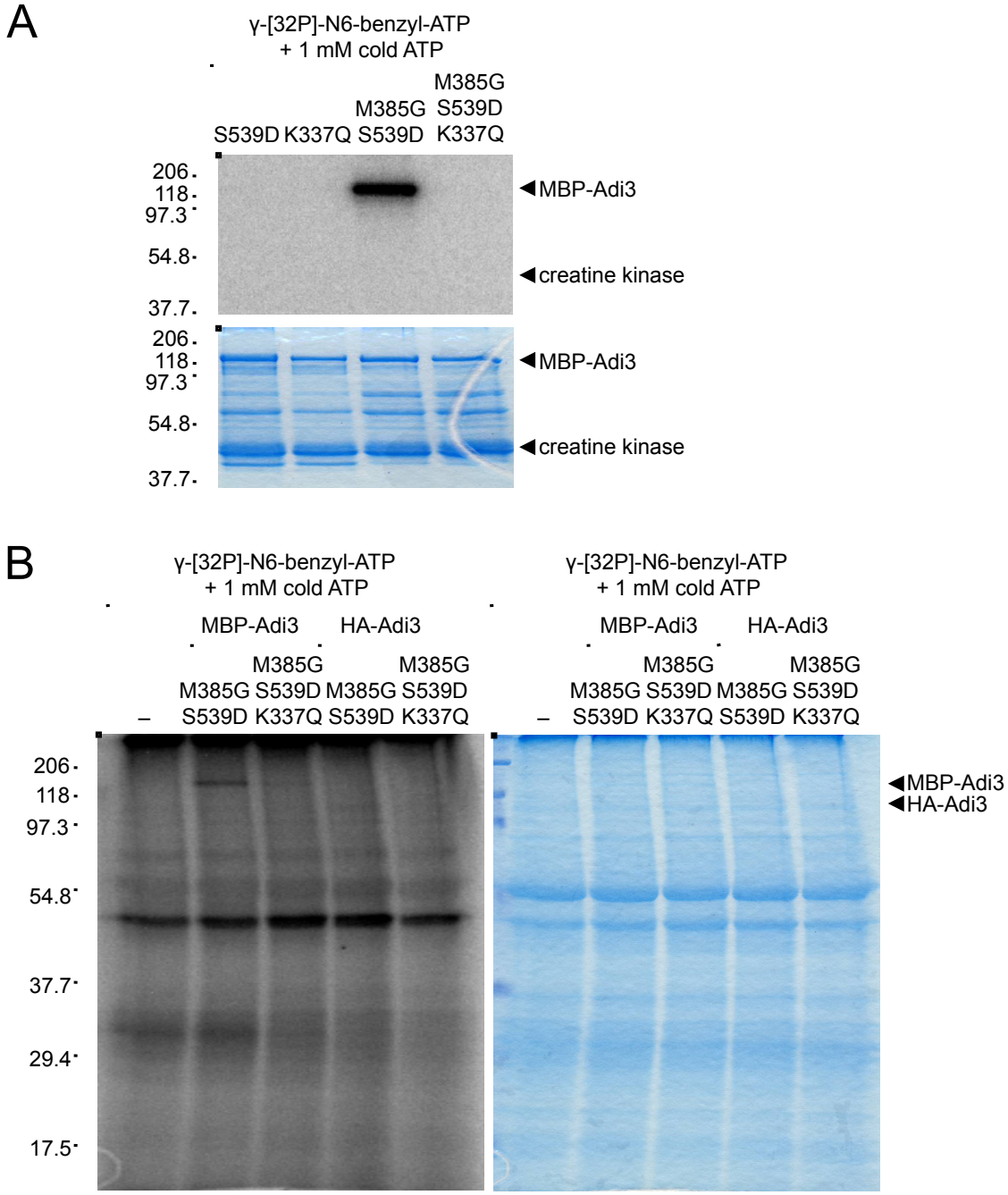

Fig. 41. Phosphorylation of tomato cell lysate with γ-[32P]-N6-benzyl-ATP in a creatine kinase ATP-regenerating buffer. A, In vitro test of creatine kinase buffer using purified proteins. Top panel, phosphorimage; bottom panel, Coomassie-stained gel. B, Attempted phosphorylation of proteins in tomato protoplast extract using the same buffer as in (A), with either 1 ug of recombinant MBP-Adi3 added to lysate, or 20 ug of HA-Adi3 plasmid transfected into protoplasts before lysis. Left panel, phosphorimage; right panel, Coomassie-stained gel.

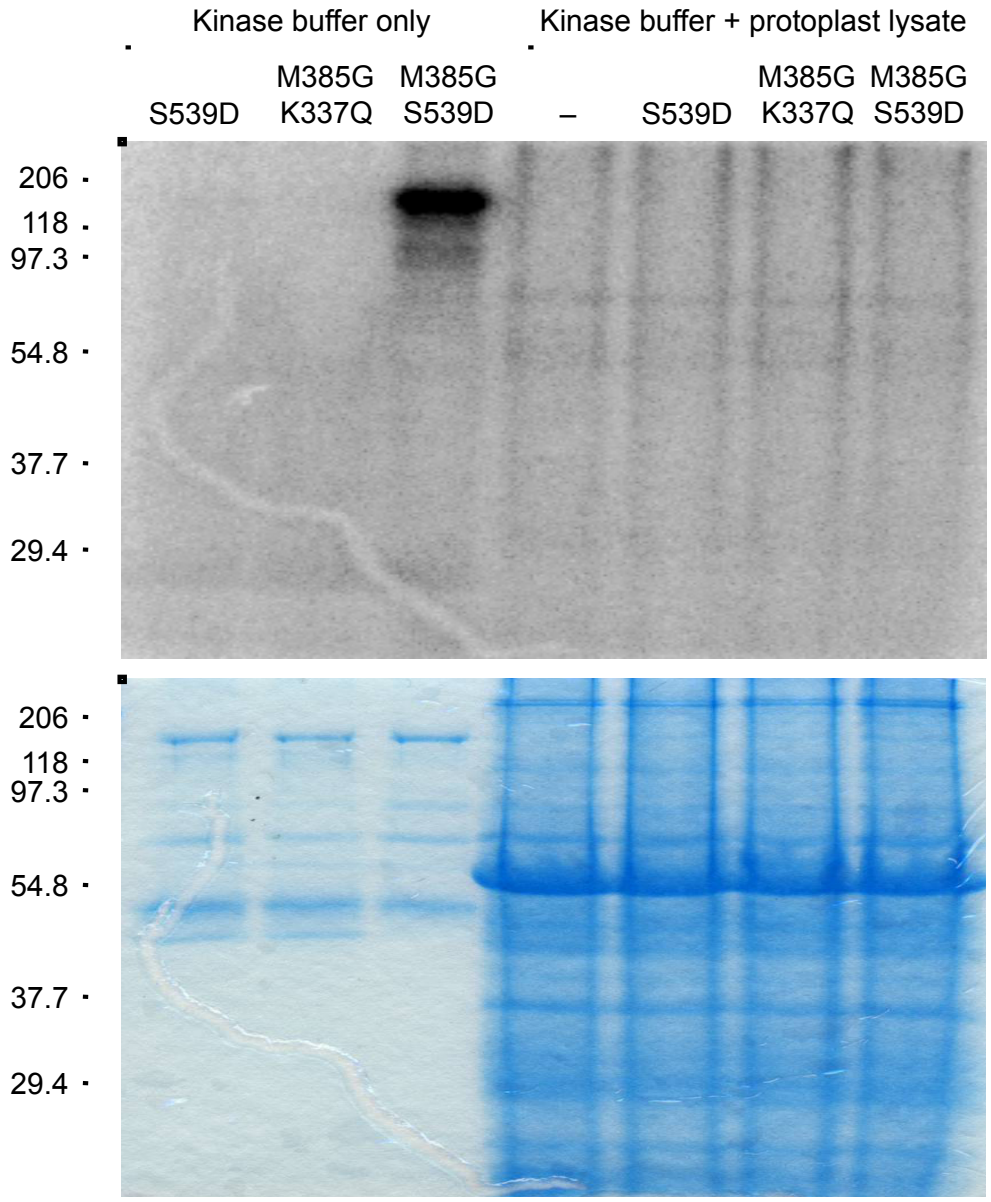

Fig. 42. Phosphorylation of tomato cell lysate with γ-[32P]-N6-benzyl-ATP in a buffer with ATPγS. Attempted phosphorylation of proteins in tomato protoplast, with 1 ug of recombinant MBP-Adi3 added to lysate. Top panel, phosphorimage; bottom panel, Coomassie-stained gel.

due to the fact that kinases often phosphorylate other kinases, and levels of these proteins are typically kept very low. Thus, even if specific phosphorylation of an Adi3 substrate occurred during my experiments, it is possible that individual phosphorylated substrates would be undetectable due to very small amounts of the substrate proteins.

6.6d Unsuccessful attempts to phosphorylate a known Adi3 substrate in vivo

Once Gal83 was identified as a substrate of Adi3, the ability of *as*-Adi3 to phosphorylate Gal83 in the presence of tomato leaf extract was investigated. Adding plant extract to Adi3 and Gal83 quickly reduced or abolished specific γ-[^{32}P] incorporation by both Adi3 and Gal83 (Fig. 41; Fig. 42; Fig. 43), regardless of the fact that wild type kinases should be unable to use γ-[^{32}P]-N6-benzyl-ATP. The fact that no groups have reported successful identification of a plant kinase substrate using radiolabeled bulky ATP analogs seems to support the conclusion that this technique is not yet feasible. In my opinion, there are several possible explanations for the decrease in specific phosphorylation by *as*-Adi3 and the nonspecific incorporation of γ-[^{32}P] in the presence of plant extracts to which γ-[^{32}P]-N6-benzyl-ATP has been added (Fig. 41; Fig. 42; Fig. 43). First, it is possible that some enzyme is capable of removing the bulky N6 group from γ-[^{32}P]-N6-benzyl-ATP, thereby producing γ-[^{32}P]-ATP that can be easily used by endogenous proteins. Second, an ATPase or other enzyme might remove the gamma phosphate from γ-[^{32}P]-N6-benzyl-ATP, either transferring it to ADP or another nucleoside diphosphate, or producing N6-benzyl-ADP and free γ-[^{32}P] inorganic phosphate. Because *as*-kinase inhibitors like 1-NA-PP1 have a bulky ring structure similar structure to the N6 substitution of N6-benzyl-ATP, but have been used

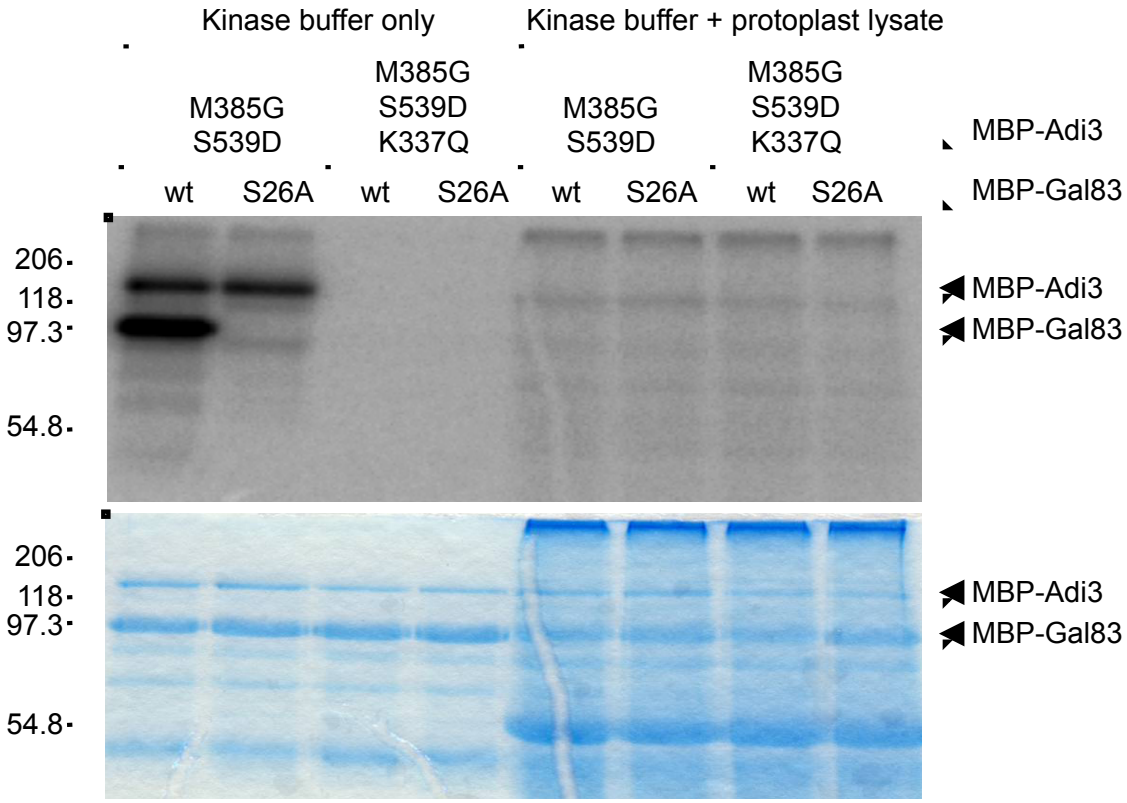

Fig. 43. Addition of tomato leaf extract abolishes phosphorylation of Gal83 by Adi3. Samples were analyzed as in Figure 2. Top panel, phosphorimage; bottom panel, Coomassie stained gel.

successfully in plants (Bohmer et al., 2011; Salomon et al., 2011), it seems more likely that the radiolabeled phosphate is somehow being removed, either by an ATPase or another plant protein whose activity we were unable to inhibit. Recently techniques have been developed to crosslink *as*-kinases with their substrates (Statsuk and Shokat, 2012), so in the future it will be interesting to test whether this method might be moresuccessful in isolating substrates of plant kinases. Another technique I did not attempt, but which could prove to be successful in the future, is to separate as-Adi3 phosphorylated plant protein extracts by two-dimensional electrophoresis. This would provide much better resolution of individual phosphorylated proteins, and in any case will probably be essential to identify potential Adi3 substrates by mass spectrometry.

CHAPTER VII

CONCLUSION AND FUTURE DIRECTIONS

7.1 Chapter III conclusions and future directions

Chapter III reported the characterization of *Pp*PDK1, a homologue of PDK1 from *P. patens*. Unlike *A. thaliana* or tomato but like rice, PDK1 appears to be a single-copy gene in *P. patens*; in contrast to all these vascular plants, *Pp*PDK1 lacks a lipid-binding domain. Nevertheless, *Pp*PDK1 could rescue the lethal phenotype of yeast lacking PDK1, and also could phosphorylate AGC kinases from tomato and *P. patens* in their activation loop. These results indicate that PDK1 function is probably broadly similar across diverse species. Mutagenesis of conserved PIF-binding pocket residues reduced *Pp*PDK1 kinase activity and substrate interaction. When *Pp*PDK1 was knocked out by homologous recombination-mediated gene replacement, the *pdk1* moss was viable but severely compromised in its growth. This study provides a foundation for beginning to dissect the conserved and lineage-specific functions of plant PDK1s. However, the experiments presented in this chapter did not fully address several aspects of *Pp*PDK1 function. I believe the following issues would be interesting to investigate in the future.

First, the ability of *Pp*PDK1 to be regulated by lipid interactions should be explored in more detail. My qualitative lipid-binding studies in Chapter III suggest that *Pp*PDK1 does not bind lipids, but to address the possibility of subtler protein-lipid interactions it will be important to assay *Pp*PDK1 interaction with, and activation by, individual species of phospholipids. One method that might be used to successfully

investigate PDK1-lipid interactions has been described previously (Anthony et al., 2004). In these experiments phospholipids conjugated to beads were used to pull down PDK1 and other lipid-binding proteins. It is also possible to study activation of PDK1 by lipids. Either protoplasts expressing PDK1 (Anthony et al., 2004) or purified PDK1 protein (Friant et al., 2001) may be treated with different lipid species; PDK1 activation by the lipids can then be measured by standard kinase assays.

Second, the phenotypes of *Pp*PDK1-6His and *Pp*PDK1^{K71A}-6His transformed moss differed noticeably from wild-type moss at later time points (Fig. 22). The most probable explanation is that improper RNA processing occurred because of the lack of a terminator sequence after the PpPDK1-6His coding sequence; thus, new gene replacement lines without this problem should be generated. I recently produced new gene replacement constructs, which have a nopaline synthase (NOS) terminator immediately 3' of the *Pp*PDK1-GFP and *Pp*PDK1^{K71A}-GFP coding sequences. I also transformed moss with these gene replacement constructs. From visual inspection of the colonies, it appears that at least some of them are indistinguishable from wild-type moss, but genotyping has not yet been performed to verify that endogenous *Pp*PDK1 is absent. I wished to generate *Pp*PDK1-GFP gene replacement lines rather than *Pp*PDK1-6His lines because of difficulties pulling down 6His-tagged protein from cell lysate. One future goal would be to immunoprecipitate the GFP-tagged protein from cell extracts and use it in kinase assays; this would provide further verification that *Pp*PDK1 activity truly differs in *Pp*PDK1-GFP and *Pp*PDK1^{K71A}-GFP gene replacement moss, and that

*Pp*PDK1^{K71A}-GFP moss is indistinguishable from wild-type moss despite having very low *Pp*PDK1 kinase activity.

Third, to ensure the phenotype of *pdk1* knockout moss is truly as I reported in Chapter III, independent lines of *pdk1* moss should be recovered. I recently transformed more moss with the *pdk1* knockout construct. From visual inspection of the colonies, it appears that some of them are unable to produce leafy gametophore tissue, and that browning of some protonemal filaments appears after more than 1 month of growth on BCD or BCDAT plates, as shown in Fig. 22. However, genotyping has not yet been performed to verify that endogenous *Pp*PDK1 is absent. Moreover, the best evidence that *Pp*PDK1 plays an important (but non-essential) role in moss growth and survival would come from complementing the *pdk1* knockout line with wild-type *Pp*PDK1. In the future it will be important to transform *pdk1* moss with a PpPDK1 gene replacement construct (probably the *Pp*PDK1-GFP-NOS terminator construct discussed above), to ensure that the wild-type phenotype is restored.

Finally, as has been done for some of the *A. thaliana* AGC kinases (Bögre et al., 2003; Martin et al., 2009; also see Introduction), the full repertoire of *P. patens* AGC kinases should be catalogued for comparison with AGC kinases from other plants. This task is not well suited to the current capabilities of the Devarenne lab, as it would require substantial bioinformatics expertise to correctly and completely evaluate the *P. patens* genome for protein kinases that fit the sequence criteria of the AGC group. However, collaboration with a group of bioinformatics experts could facilitate generation of a list of *P. patens* AGC kinases; ideally several other model plants would also be considered

(e.g. rice, the fern relative *S. moellendorffii*, and algae such as *C. reinhardtii* and *C. merolae*). It would be interesting to compare the AGC kinases of different plant species to better understand the kinases that are likely to perform conserved tasks versus lineage-specific tasks. A similar analysis was performed with the entire kinomes of human, *S. cerevisiae*, *C. elegans*, and *D. melanogaster* (Manning et al., 2002a); this report greatly illuminated our understanding of kinase evolution. To my knowledge no such work has been performed in plants; one study compared the *A. thaliana* and *S. cerevisiae* kinomes (Wang et al., 2003), but a comparative analysis of multiple photosynthetic species is lacking. Because AGC kinases are the focus of research in the Devarenne lab, a catalogue of plant AGC kinases would be more immediately relevant to our work, but plant biology in general would greatly benefit from a systematic analysis of several plant kinomes.

If a comprehensive list of *P. patens* AGC kinases can be generated, it would be possible to knock out each one (particularly those also found in other plants) to study the impact on moss growth, survival, stress responses, development, and reproduction (for example, the *pdk1* knockout moss appears to be unable to produce spores). As mentioned in the introduction, one of the advantages of *P. patens* as a model plant is its ability to perform high efficiency homologous recombination, thus eliminating the dependence on the random T-DNA insertion libraries that are commonly used to study loss-of-function alleles in *A. thaliana*. Another possible line of investigation would be to measure the ability of *Pp*PDK1 to phosphorylate and activate each of the *P. patens* AGC kinases, as was previously done with the *A. thaliana* AGC VIIIa kinases (Zegzouti et al.,

2006b). It is unfortunate that Zegzouti et al. did not also analyze the 16 kinases belonging to other AGC subfamilies; perhaps a side-by-side study of PDK1 activation could be performed with the *A. thaliana* and *P. patens* AGC kinases. In my opinion, it is a shame that most plant AGC kinase research seems to focus on the AGC VIII subfamily; in the future I hope to learn more about the functions of the other subfamilies.

7.2 Chapter IV conclusions and future directions

In Chapter IV I compared putative PDK1 sequences from a number of distantly related photosynthetic and non-photosynthetic organisms and discussed possibilities for PDK1 function and evolution in eukaryotes. Though almost all experimentally confirmed PDK1s quite closely resemble the human PDK1, I discovered a relatively high degree of diversity in PDK1 size (ranging from approximately 300 to 2000 amino acids) and protein domain architecture. Particularly striking was the lack of a lipid-binding PH domain in some of the PDK1 homologues, including species of fungi, algae, and diatoms. This is surprising because, with the possible exception of *P. patens* PDK1, all PDK1s reported so far are regulated (in activity, localization, or both) by lipid interactions. My phylogenetic analysis results suggest that PDK1 evolutionary history is probably quite complex, and the relative diversity of the PDK1 sequences may hint at a number of intriguing functional differences that have not yet been explored.

It appears to be a strange coincidence that most model organisms in which PDK1 has so far been investigated have strikingly similar PDK1s, both in protein size and in domain architecture. As the cost and technical challenges of high throughput sequencing continue to decrease at an astounding rate, I expect that dozens to hundreds of new

eukaryotic genome sequences will be released in the next few years. Groups like Gerard Manning's laboratory will scrutinize the kinomes of some of these organisms. Together, genome and kinome analyses published in the near future should give us a much better picture of the evolution of eukaryotic protein phosphorylation. In particular I am interested in learning more about the evolutionary history of PDK1. To do this definitively requires a large number of PDK1 sequences from closely related organisms. Once the sequences have been obtained, they are aligned and sequence changes are quantified. Ancestral states can then be inferred to arrive at a putative history; with enough carefully chosen organisms it might be possible to find points when lipid-binding domains were clearly gained or lost. Currently, most organisms do not have many close relatives with fully sequenced genomes. Within plants, *A. thaliana* has by far the largest number of fully sequenced close relatives. However, as the PDK1 sequences of all vascular plants appear to be highly similar (Fig. 28), a clade of vascular plants is probably not an interesting candidate for a PDK1 evolutionary study. If genome sequencing and analysis technologies continue to progress at the rate they have for the past decade, before long it might be possible to find an evolutionarily interesting transition point (e.g. between nonvascular and vascular plants, or between animal and fungal lineages) with a large number of fully sequenced, closely related species. In lieu of that, PDK1 seqeunces must be obtained from organisms without complete genome sequences; this is typically done using degenerate PCR primers. Again, this lies outside the current area of expertise for the Devarenne lab, but it could be pursued in the future.

One problem with searching for genes (e.g. PDK1) in an organism without a fully sequenced genome is that it is difficult to conclusively determine how many copies of the gene are present. For example, *T. vaginalis* and *L. major* appear to possess 5 and 3 putative PDK1 homologues, respectively, that differ quite drastically from each other (Manning et al., 2011); even with carefully designed primers one or more of these sequences might not have been recovered by degenerate PCR. It is intriguing to imagine that some organisms could have unusually expanded and diverse PDK1 gene families. As mentioned near the end of Chapter IV, perhaps multiple instances of gene duplication and subsequent divergence helped generate the impressive PDK1 diversity seen in Fig. 28 and 29. I certainly would be interested in testing this hypothesis in the future. The first task would be to perform yeast complementation and kinase activity assays to determine which genes annotated as PDK1 are true functional homologues, i.e. which ones can phosphorylate known AGC kinase substrates in the activation loop. Next, the verified PDK1 homologues from close relatives should be compared to understand which genes are likely to have the closest relationship, and thus have a common origin. From this analysis it should be possible to form a plausible picture of PDK1 gene duplication; finally the possible functional divergence of duplicated genes can be addressed by studying the expression, activity, localization, and regulation of each gene.

One final line of investigation I would be interested in pursuing is to try to understand why so many PDK1s are apparently dramatically different from each other. Why should some organisms (e.g. *P. patens*, several green algae, and at least one fungus) have a PDK1 of 300-350 amino acids that is basically nothing more than a

kinase domain (category 5 in Fig. 29), whereas others (e.g. at least two green algae and the majority of fungi) have relatively enormous PDK1s of >800 amino acids, with large regions of unknown function on one or both sides of the kinase domain (categories 6-8 in Fig. 29)? There must be important differences in PDK1 function and/or regulation that no one has yet reported. The category 5 PDK1 (Fig. 29) is reminiscent of the catalytic subunit of PKA, both of which consist of little more than a kinase domain; but PKA is inhibited by the regulatory subunit until the presence of cyclic AMP induces catalytic subunit release (Pearce et al., 2010). No analogous process has been described for any PDK1 homologue, so there is no information yet about how the activity of a very small (presumably consitutively active) PDK1 could be regulated. Possible mechanisms of regulation include transcriptional, post-transcriptional, or post-translational control. Both PDK1 and its substrates could be likely candidates for either transcriptional or post-transcriptional regulation, but post-translational control of a very small PDK1 (as opposed to regulation of substrate proteins) would most likely have to occur through novel interactions with the PDK1 kinase domain. On the other hand, the very large PDK1s (categories 6-8 in Fig. 29) present no shortage of possibilities for post-translational regulation. The majority of these large regions of sequence outside the kinase domain have no annotated function; thus, investigating their roles in PDK1 activity or regulation could be challenging. I would approach this issue by performing initial experiments with genetically tractable organisms, in which it is possible to create stable knockout and transgenic lines. The easiest experiments to perform would be to analyze deletions of different portions of PDK1 for a visible phenotype. Another

possibility is to search for proteins that interact with immunoprecipitated tagged PDK1 (both full length and deletions).

7.3 Chapter V conclusions and future directions

Chapter V presented results that suggest the existence of a second PDK1 phosphorylation site on Adi3, at Ser212, which lies outside the kinase domain. This site was originally identified in the *A. thaliana* sequence homologue of Adi3, AGC1-3 (Gray and Devarenne, 2012). I confirmed that PDK1 also phosphorylates Adi3 at this site in vitro, and that while phosphorylation does not appear to alter Adi3 autophosphorylation, it may increase Adi3's activity on a substrate approximately 2 fold. Finally, I show that it may be possible to resolve individual differentially phosphorylated forms of Adi3 using protein gels with reduced ratios of bisacrylamide crosslinker. These results do not conclusively demonstrate PDK1 phosphorylation on Ser212, but they provide tools with which to further investigate this potential phosphorylation event.

Two important future steps must obviously be taken before the biological significance of Ser212 phosphorylation can be gauged. First, we must conclusively determine if PDK1 phosphorylates Ser212 in vivo. Second, we must assess whether altering the phosphorylation state of Ser212 produces a phenotype (e.g. the ability of Adi3 to negatively regulate PCD). In my opinion, tomato is not the ideal system in which to carry out either of these experiments for several reasons, foremost of which is that stable knockout and transgenic lines are not easily produced. It would be simpler and less time consuming to initially perform both tests with AGC1-3 and PDK1 in *A. thaliana*, then confirm the results with Adi3 in tomato if they are promising.

My first step would be to generate a homozygous T-DNA knockout line for AGC1-3; if this were not possible due to lethality, I would transform a heterozygous *agc1-3* T-DNA line with tagged versions of wild-type, K392Q (kinase inactive, analogous to Lys337 in Adi3), S269A (analogous to Ser212 in Adi3), S596A (analogous to Ser539 in Adi3), and S269A/S596A versions of AGC1-3. I would then self the plants to obtain progeny that express the tagged version of AGC1-3 but are homozygous for knockout of the endogenous AGC1-3. Mass spectrometry could be performed on AGC1-3 immunoprecipitated from these lines to unambiguously determine whether Ser269 and Ser596 are phosphorylated. To ensure that this phosphorylation event is due to PDK1 activity, each transgenic line could be crossed with the *pdk1-1*/*pdk1-2* knockout line generated previously (Camehl et al., 2011), and the immunoprecipitation and mass spectrometry repeated. To assess the phenotypic effects of Ser269 and Ser596 phosphorylation, the AGC knockout line (if viable) and each line expressing only a tagged version of AGC1-3 can be subjected to various stresses, such as pathogen challenge, heat, or salt stress. These treatments can be performed with whole seedlings or plants, or with protoplasts produced from leaf tissue. Cell viability can be tested for each plant, and if Ser269 or Ser596 must be phosphorylated for full AGC1-3 functionality, then the Ser to Ala mutants will have decreased viability and/or stress tolerance. If the results of these experiments suggest an important role for Ser269, then further investigation of Adi3 Ser212 could continue.

7.4 Chapter VI conclusions and future directions

In Chapter VI I discussed my attempts to produce a form of Adi3 that would be suitable for substrate searches using bulky ATP analogues. This method has been used successfully to identify direct substrates of a number of mammalian and yeast protein kinases. My work identified a "gatekeeper" amino acid that enables Adi3 to selectively use a bulky ATP analogue, and also showed that a known substrate of Adi3 could be phosphorylated by analogue-sensitive Adi3 in vitro. Thus, in principle analogue-sensitive Adi3 should be perfectly suited to a substrate search using plant tissue extracts. Unfortunately, many attempts to transfer this system to tomato leaf extracts were unsuccessful. I speculated that plants might possess an ATPase or other enzyme that is capable of removing the gamma phosphate from ATP, resulting in high background phosphorylation and/or low analogue-sensitive kinase phosphorylation.

The future of this project is uncertain. In principle it is an elegant and highly effective way to specifically target direct kinase substrates, yet plant tissue extracts seem to pose a difficult challenge for the use of radiolabeled ATP analogues. As with the previous section, my next step would be to search for substrates in *A. thaliana* using an analogue-sensitive version of AGC1-3, then verify the results for Adi3. I would first transform a heterozygous *agc1-3* T-DNA line with a tagged analogue-sensitive AGC1-3, and then self the plants to obtain progeny that express the analogue-sensitive AGC1-3 but are homozygous for knockout of the endogenous AGC1-3. These plants can be treated with the analogue-sensitive kinase inhibitor 1-NA-PP1. Unfortunately this method results in a loss of phosphorylation signal in AGC1-3 substrates, rather than a

gain as the radiolabeled analogue method does. However, until the problem of analogue degradation by plant extracts is circumvented, this seems most likely to succeed.

7.5 Overall conclusions

My experiments have provided insight into two members of the AGC group of protein kinases, one (PDK1) that is conserved in all eukaryotes and one (Adi3) that appears to be present only in plants. These experiments give a new perspective in our view of plant AGC kinase function and regulation. In conclusion, it is an exciting time to study protein kinases and protein phosphorylation. What developments might the future hold? As we increasingly recognize signal transduction as web-like rather than linear, the assessment of global protein phosphorylation will be an important tool. The use of high throughput technologies like genomics and proteomics should enable an unprecedented breadth and depth of investigation into the myriad phosphorylation-based signaling pathways that are a characteristic feature of eukaryotic life.

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